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ADRENOCORTICAL FUNCTION AND CORTISOL METABOLISM IN OLD AGE*

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ABSTRACT

In this study the influence of old age on adrenocortical function and cortisol metabolism was investigated, along with renal, hepatic and thyroid function in 15 young men (25–40 years old) and 50 old men (60–96 years old). By the criteria of 8-a.m. plasma levels of free and conjugated 17-hydroxycorticosteroids (17-OH-CS) and the plasma corticoid response to ACTH stimulation, no significant change in adrenocortical function could be demonstrated with advancing age. However, there was a progressive and significant decrease in the urinary excretion of 17-OH-CS with aging. Following intravenous administration of a standard dose, cortisol disappeared from the circulation at a significantly slower rate in the old men. The maximal levels of plasma conjugated 17-OH-CS observed after cortisol infusion were statistically the same for all ages, but the rate of decay from peak values was slower in the older subjects. These alterations in cortisol metabolism were associated with a significant increase in the level of blood urea nitrogen and bromsulfalein retention, and a significant decrease in endogenous creatinine clearance and basal metabolic rate. The plasma protein-bound iodine level and thyroidal uptake of I^{131} were unaffected by aging. A combination of decreased cortisol catabolism by the liver and other tissues and an impairment in the renal excretion of cortisol metabolites is proposed to explain these observations. The thyroid gland does not appear to be involved.

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THE early demonstration that the excretion of neutral 17-ketosteroids declines with advancing age in women as well as men first led to the speculation that adrenocortical function might be decreased in old age. Subsequent fractionation studies revealed that this decrease in urinary 17-ketosteroids is due primarily to a lower excretion of 11-deoxy-17-ketosteroids, with little change in the excretion of 11-oxy-17-ketosteroids and other cortisol metabolites (1–3). On the basis of these results it would appear that corticosteroid production declines little with advancing age, whereas androgen production by both the adrenal cortex and the testis decreases considerably.

Other lines of investigation have suggested that adrenocortical function and metabolism might be altered in old age. Samuels *et al.* (4, 5) and Tyler *et al.* (6) reported greater than normal rises in plasma 17-hydroxycorticosteroid (17-OH-CS) levels in human subjects over the age of 55 following the administration of corticotropin (ACTH) intravenously. The results of these ACTH tests were not interpreted as demonstrating adrenocortical hyperfunction because the apparent hyper-response to ACTH was associated with a decreased rate of removal of cortisol from the circulation following intravenous administration.

The mechanisms involved in this reduced rate of cortisol utilization in old age are unknown. The observations that cortisol utilization is decreased in patients with kidney and liver disease and in hypothyroidism (7–10) might be relevant, especially in view of reports that a decrease in thyroid and kidney function occurs with old age in the absence of overt clinical disease (11). The present study was undertaken to ascertain whether decreased cortisol utilization in old age is associated with impaired function of the liver, kidney or thyroid gland and to investigate other parameters of adrenocortical function in the aged.

MATERIALS AND METHODS

The older subjects in this study were judged to be free of endocrine, renal and hepatic disease by conventional clinical and laboratory examination. None was acutely ill or febrile, and none had recently been subjected to stressful procedures. All were males. They were arbitrarily divided into three groups by decades over 60 years of age. There were 22 subjects in the 60–69 year age group (average age, 63 years); 14 in the 70–79 year group (average age, 73 years); and 14 in the group over 80 years of age (average age, 83 years). The control group consisted of 15 normal subjects ranging in age from 25 to 40 years (average, 33 years).

Standard infusions of cortisol (1 mg. per Kg.) were administered intravenously as described previously (8). Blood samples were routinely drawn at one, two, four and six hours after the beginning of the infusion, and the concentration of free 17-OH-CS in the plasma was determined by a modification of the method of Nelson and Samuels (12). Levels of conjugated 17-OH-CS in the plasmas were measured by a modification of the method of Bongiovanni (13).

The half-lives for cortisol utilization and the apparent volumes of distribution of the administered cortisol were calculated as described by Samuels *et al.* (5). These calculations are based on the observation that a semilogarithmic plot of the plasma 17-OH-CS concentration at intervals following the intravenous administration of cortisol assumes a linear function from one to eight hours after administration.

The ACTH stimulation tests were performed by administering 25 units of ACTH intravenously over a six-hour period and measuring the levels of plasma 17-OH-CS at zero, one, two, four and six hours after the start of the infusion (14).

Urinary 17-OH-CS excretion was measured by a modification of the methods of Glenn and Nelson (15) and Bongiovanni (13) which have been described in detail in a previous publication (9). All methods for 17-OH-CS used in this study utilize the Porter-Silber reaction for quantitation; this reaction measures all C-21 steroids with the 20-keto-17,21-dihydroxy configuration.

Thyroid function was estimated by measuring the basal metabolic rate (BMR), plasma protein-bound iodine level (PBI), and the thyroidal uptake of radioactive iodine at twenty-four hours, by conventional methods.

Bromsulfalein (BSP) retention at forty-five minutes after administering 5 mg. per Kg. of body weight was used to estimate liver function. Subjects with clinical findings of liver disease and/or a BSP retention greater than 10 per cent were excluded from the study. Renal function was evaluated by serum creatinine and blood urea nitrogen (BUN) levels and endogenous creatinine clearance, using routine laboratory procedures for the chemical determinations.

RESULTS

The results of the tests of adrenocortical function in the various age groups are shown in Table 1. Although there was a progressive increase in the 8-a.m. level of free plasma 17-OH-CS for each older age group, the increase was small and not significantly different from that for the control group at the 95 per cent level of probability. The same observation applies to the 8-a.m. levels of conjugated plasma 17-OH-CS for the different age groups. The daily urinary excretion of 17-OH-CS fell with increasing age.

TABLE 1. INFLUENCE OF AGING ON ADRENOCORTICAL FUNCTION AND THE METABOLISM OF CORTISOL

Test	A 25–40 yrs.	B 60–69 yrs.	C 70–79 yrs.	D Over 80 yrs.	Significant differences between groups*
Free plasma 17-OH-CS at 8 a.m. (μg./100 ml.)	12.4±1.1**	12.7±1.1	14.8± 1.9	14.5±1.8	None
Conjugated plasma 17-OH-CS at 8 a.m. (μg./100 ml.)	12.5±2.0	9.7±1.5	15.8± 3.0	16.2±3.3	None
Urinary 17-OH-CS (mg./24 hrs.)	8.5±0.4	6.1±0.6	5.3± 0.6	3.9±1.6	A *vs.* B = .01 >P>.001 A *vs.* C = P<.001 A *vs.* D = .02>P>.01
Half-life for cortisol utilization (min.)	112.0±5.1	144.0±7.3	154.0±11.2	169.0±8.8	A *vs.*B = .01>P>.001 A *vs.* C = .01>P>.001 A *vs.* D = P<.001 B *vs.* D = .025>P>.01
Apparent volume of distribution of infused cortisol (% body weight)	81.0±3.3	92.0±4.7	94.0± 7.3	92.0±6.7	None

* Determined by the Student *t* test. For "none," P>.05.
** Mean and standard error of the mean.

The differences in 17-OH-CS excretion between each of the older age groups and the young control group were highly significant, with a probability of less than 2 per cent that the results were due to chance (Table 1).

Old men and young men responded equally well to the administration of ACTH. The young control group had a mean 8-a.m. plasma 17-OH-CS level of 12.1 ±1.1 μg. per 100 ml. (SEM)[1] which rose to 41 ±3.2 μg. at six

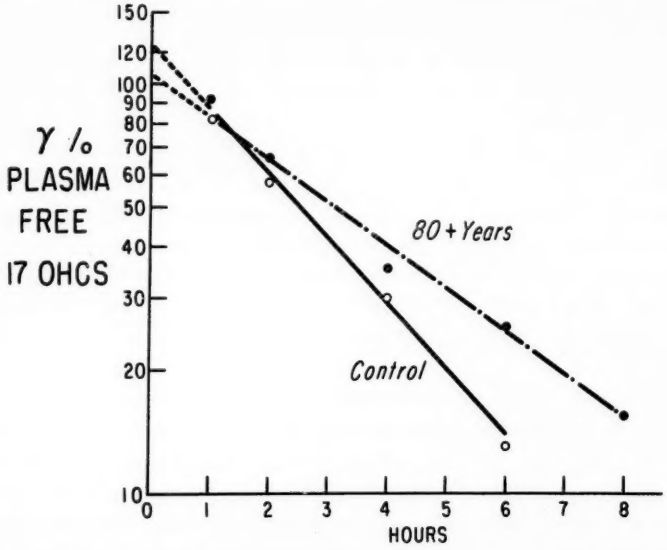

FIG. 1. Free plasma 17-OH-CS levels at intervals following cortisol infusion in young and old men.

hours after the start of the intravenous ACTH infusion; the mean increase was 29 μg. per 100 ml. In 9 subjects between the ages of 72 and 96 years, the mean control level for plasma 17-OH-CS was 15.3 μg. per 100 ml., with an increase to 44.6 μg. at six hours, an increment of 29.3 μg. per 100 ml. The mean increases in 17-OH-CS levels with the ACTH tests were not significantly different between the two groups.

Following a standard intravenous infusion of cortisol, a semilogarithmic plot of free plasma 17-OH-CS levels against time was linear from one to eight hours after the infusion in all age groups, with a progressive decrease in slope with each decade in the older age groups. The cortisol decay curves for the control group and those over 80 years of age are illustrated in Figure 1.

A progressive decrease in the rate of removal of cortisol from the circula-

[1] Mean and standard error of the mean.

tion following intravenous infusion was observed with aging. The half-life for cortisol was 112 ±7.3 minutes[1] in the control group, 144 ±7.3 minutes in the 60–69 year group, 154 ±11.2 minutes in the 70–79 year group, and 169 ±8.8 minutes in those more than 80 years old. Testing for significant differences in the mean half-lives for cortisol between the various age groups by the Student *t* test revealed a high degree of significance between the control group and the group over 80 years old ($P < .001$), the control group versus the 70–79 year group ($.01 > P > .001$), and the control group versus the 60–69 year group ($.01 > P > .001$).

The apparent volumes of distribution of the infused cortisol, calculated by extrapolating the plasma cortisol concentration back to zero time, were

TABLE 2. CONJUGATED PLASMA 17-OH-CS LEVELS FOLLOWING INFUSION OF CORTISOL

Age group	Plasma conj. 17-OH-CS (μg./100 ml.)						
	Hours after infusion of corticol						
	0	½	1	2	4	6	8
Control (25–40 yrs.)	12.5±2.0*	37.3±3.6	59.5±7.6	69.4±12.4	53.3±7.2	41.4±7.7	
60–69 yrs.	9.7	33.1	47.2	52.4	46.7	36.2	27.5
70–79 ys.	15.8	35.0	50.8	56.5	51.5	44.2	37.8
80–89 yrs.	16.2	36.8	57.4	62.9	61.9	55.7	46.4

* Mean and standard error of the mean.

not significantly different between any of the age groups, falling between 81 and 94 per cent of the body weight.

Following infusion of cortisol the level of conjugated 17-OH-CS in the plasma rose to a peak at two hours in the control group and then decayed in exponential fashion until six hours after the infusion (Table 2). In the old-age groups there was a slight delay in the time of attaining the peak level, but the maximal levels achieved were not significantly different from those for the control group. The rate of removal of the conjugated 17-OH-CS from the circulation was not logarithmic in any of the older groups up to eight hours after infusion, and was increasingly slower with each higher decade. A semilogarithmic plot of plasma conjugated 17-OH-CS levels against time for the control group and the oldest group, which illustrates this difference most strikingly, is shown in Figure 2.

In an attempt to evaluate liver function, BSP retention tests were carried out on all the subjects in this study. As shown in Table 3, a slight but significantly greater retention of BSP was observed in the 70–79 year group and in the group over 80 years old when mean values were compared with those for the control group, but not in the 60–69 year group.

Plasma BUN and creatinine levels and endogenous creatinine clearances were determined, to evaluate renal function. A significant difference in mean plasma BUN levels was found between each of the three old-age groups and the young control group (Table 3). Plasma creatinine levels did

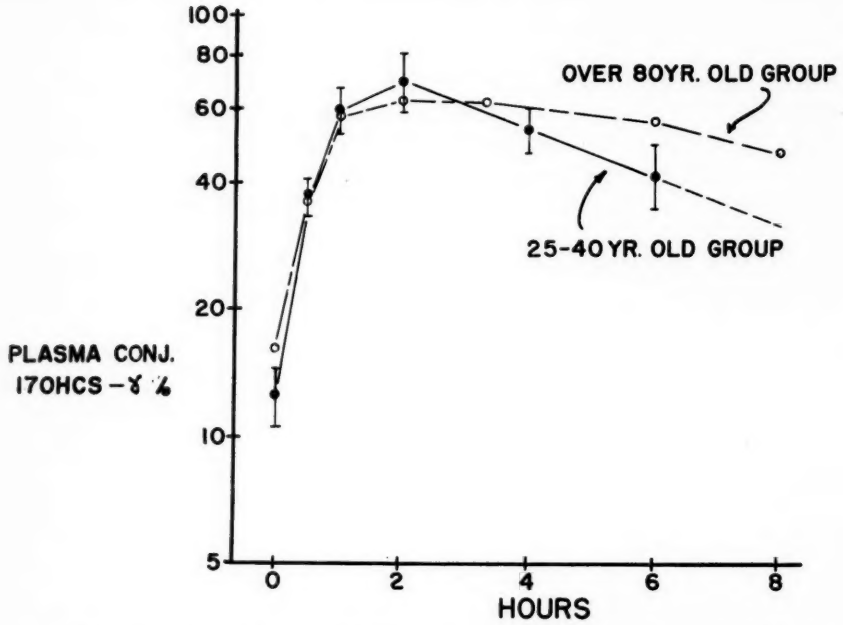

FIG. 2. Conjugated plasma 17-OH-CS levels at intervals following cortisol infusion in young and old men. The vertical lines with bars indicate ± one standard error of the mean (SEM).

TABLE 3. INFLUENCE OF AGING ON THYROID, KIDNEY AND LIVER FUNCTION

Test	A 25–40 yrs.	B 60–69 yrs.	C 70–79 yrs.	D Over 80 yrs.	Significant differences between groups*
PBI (μg./100 ml.)	5.4± 1.0**	5.6±0.3	6.2±0.6	6.7±1.3	None
Thyroidal I^{131} uptake (% dose)	30.0± 4.1	28.6±4.1	26.0±3.3	26.1±3.0	None
BMR (cals./sq. m./hr.)	40.2± 0.6	36.3±0.9	35.0±1.2	32.3±2.2	A *vs.* B = P < .001 A *vs.* C = P < .001 A *vs.* D = .01 < P < .001
Plasma BUN (mg./100 ml.)	11.7± 0.8	16.1±1.2	16.1±0.9	20.0±1.3	A *vs.* B = .01 > P > .001 A *vs.* C = P < .001 A *vs.* D = P < .001
Serum creatinine (mg./100 ml.)	1.5± 0.13	1.5±0.09	1.6±0.12	1.9±0.14	None
Creatinine clearance (ml./min.)	100.0±11.5	64.0±3.6	45.0±6.4	30.0±1.6	A *vs.* B = None A *vs.* C = P < .001 A *vs.* D = P < .001
BSP retention (%)	3.3± 1.0	4.5±1.5	6.6±1.2	7.2±1.1	A *vs.* B = None A *vs.* C = .05 > P > .02 A *vs.* D = .02 > P > .01

* Determined by the Student *t* test. For "none," P > .05.
** Mean and standard error of the mean.

not increase significantly with old age. Except for the 60–69 year group, mean endogenous creatinine clearance was significantly reduced with increasing age.

To explore the possibility that "subclinical hypothyroidism" might be involved in the decreased utilization of cortisol in old age, the BMR, plasma PBI level and 24-hour thyroidal I^{131} uptake were measured. A

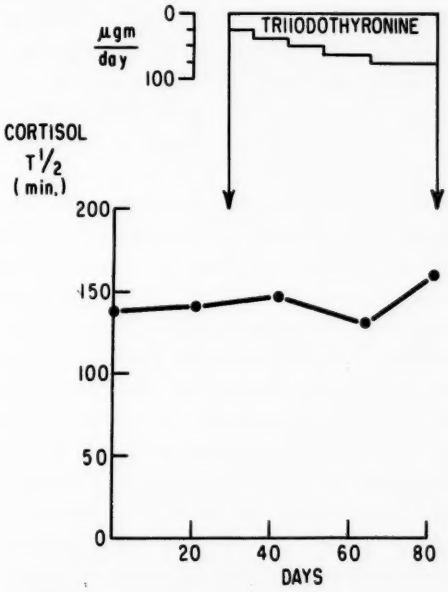

FIG. 3. The effect of administration of triiodothyronine on the utilization of cortisol in an 84-year-old man. $T\frac{1}{2}$ = half-life in minutes.

progressive decrease in BMR was observed with each older group, and this decrease was highly significant statistically. However, there was no significant alteration in the uptake of I^{131} or in the plasma PBI level with aging. As a therapeutic test, an 83-year-old patient was given triiodothyronine in doses up to 75 μg. daily for fifty-three days. No significant alteration in the rate or removal of intravenous cortisol was observed (Fig. 3).

DISCUSSION

Our data demonstrate that, following intravenous administration, cortisol is removed from the circulation at a progressively slower rate with advancing age. By the criteria of 8-a.m. free and conjugated plasma 17-OH-CS levels and the plasma corticoid response to ACTH stimulation, it might appear at first that adrenocortical function is unaffected by aging.

However, the normal rise in the concentration of plasma 17-OH-CS during ACTH stimulation suggests that the adrenal cortex becomes less responsive with aging, if the decreased rate of cortisol utilization in the older age groups is taken into account. The significant decrease in the daily excretion of urinary 17-OH-CS, along with the normal level of 8-a.m. conjugated plasma 17-OH-CS, suggests that the adrenal cortex produces corticoids at a slower rate under resting conditions in old age.

This study failed to establish an entirely satisfactory explanation for the inhibitory effect of aging on cortisol utilization. A possible renal mechanism was suggested by the elevated BUN levels, the decreased endogenous creatinine clearances, and the lower urinary excretion of 17-OH-CS in the older age groups. These findings are consistent with the reports by earlier investigators that the aging process is associated with a decrease in glomerular filtration and renal blood flow in the absence of obvious kidney disease (11). Pathology studies in old age have revealed renal atrophy, cortical infiltration with collagen, and a reduced number of functioning nephrons (16). Whether these changes are due to the aging process *per se* or to undetected kidney disease is not clear.

The decreased rate of clearance of conjugated 17-OH-CS from the circulation following infusion of cortisol (Fig. 2) and the decreased urinary excretion of 17-OH-CS can be explained on the basis of a renal mechanism. Others have shown that there is a close correlation between the glomerular filtration rate and the excretion of 17-OH-CS (17). However, the observation that the maximal levels of conjugated 17-OH-CS attained after infusion of cortisol were essentially the same for all age groups, with a time lag in reaching the peak in the older groups, suggests that there must be a decreased rate of metabolism elsewhere in the body before renal excretion can become limiting in the over-all utilization of the infused cortisol.

From a quantitative viewpoint the liver appears to be the most important site in the body for the catabolism of cortisol and the formation of conjugated metabolites which are then excreted by the kidney. The association between decreased cortisol utilization and BSP retention in the older subjects suggests that the liver might be involved. Previous reports from this laboratory have demonstrated that the utilization of cortisol correlates well statistically with retention of BSP in patients with liver disease (7), but in the aged patients the impairment in cortisol utilization is out of proportion to the degree of BSP retention. No unequivocal evidence was obtained which indicated that the liver accounts for impaired cortisol utilization in old age.

Two different mechanisms involving the liver could conceivably explain the decreased rate of cortisol utilization in old age: 1) decreased activity in the enzyme systems which metabolize cortisol within hepatic cells, or 2)

decreased availability of the infused cortisol to the intracellular enzyme systems due to a decrease in hepatic blood flow or membrane permeability. The BSP tests failed to shed any light on these possible mechanisms, since BSP retention is also influenced by hepatic blood flow, membrane permeability, and enzymatic activity.

At the intracellular level the decrease in cortisol utilization could be due to a decrease in the concentration of cofactor. Reduced triphosphopyridine nucleotide (TPNH) functions as a cofactor for the Δ^4-3-ketosteroid reductases in the liver which play a major role in the conversion of cortisol to the tetrahydro metabolites. The major source of TPNH in the liver is the hexose monophosphate (HMP) shunt. In man, an increased rate of cortisol utilization is observed in hyperthyroidism (10), hyperinsulinism with hypoglycemia (18), obesity (19), following epinephrine administration (18), and in children (20). In each of these conditions an accelerated turnover of glucose occurs which could result in an increased production of TPNH through the HMP shunt, to explain the increased cortisol utilization. Conversely, a decrease in glucose metabolism with a deficiency in TPNH production could account for the lower rate of cortisol utilization which occurs in hypothyroidism (10), liver disease (7, 8), renal disease (9), and semistarvation (21). The decrease in cortisol utilization with semistarvation in the latter study was associated with a decrease in the BMR from 17 to 21 per cent. In old age, the decreased utilization of cortisol might also be explained on the same basis if the decline in BMR with aging truly reflects a lower rate of metabolic activity in the liver, particularly in the metabolism of glucose via the HMP shunt.

It has been demonstrated that tissues other than the liver can metabolize cortisol, although not as completely (22). So far, enzyme systems for the reduction of the 3-ketone in ring A and conjugation have been demonstrated only in liver tissue. Nevertheless, it is conceivable that a defect in cortisol metabolism in extrahepatic tissues could produce the decreased rate of cortisol utilization observed in old age.

The functional status of the thyroid gland is also known to have an influence on cortisol utilization. In patients with hypothyroidism it has been demonstrated that cortisol is removed from the circulation at a significantly slower rate than in normal subjects (10). No good evidence was obtained in the present study for suspecting that the thyroid gland is involved in the decreased rate of cortisol utilization in old age. A progressive decrease in BMR was observed with advancing age, comparable to that noted by Boothby and Sandiford (23). However, the observation that thyroidal I^{131} uptakes and plasma PBI levels were statistically the same for all age groups and that the rate of cortisol utilization did not change with triiodothyronine therapy suggests that the decrease in BMR in old age is

independent of thyroid function. Of possible significance is the observation that there is a more rapid utilization of cortisol in children who have a relatively high BMR not related to thyroid function (20).

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CHANGES IN SERUM LIPIDS AND URINARY KETOSTEROIDS DURING ORAL AND INTRAMUSCULAR ADMINISTRATION OF ANDROSTERONE*†

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ABSTRACT

Androsterone administered orally to 13 men with coronary heart disease in a dosage of 100 mg. daily for one month had practically no clinical or serum lipid-shifting effects. In contrast, androsterone given intramuscularly to 8 men in a dosage of 50 mg. daily for one to four weeks resulted in a significant decrease in the levels of serum triglycerides and phospholipids, as well as total cholesterol, reflecting a reduction in the β-lipoprotein fraction. Despite this decrease, the frequency of local and systemic side-effects renders this compound unsuitable for routine clinical use. Androsterone was excreted in the urine chiefly as the glucuronide, and a small amount of *iso*androsterone was also isolated.

ALTHOUGH androsterone (androstan-3α-ol-17-one) was the first crystalline androgen isolated, and research on its preparation and physiologic effects vigorously pursued between 1931 and 1934, the isolation of the far more active compound, testosterone, from bull testis by Laqueur's group in 1935 resulted in less interest in the study of androsterone. Relatively little importance was attached to this weak androgen, except as one of several major excretory products of the hormones of the adrenal cortex and testis, until Hellman and colleagues at the Sloan-Kettering Institute in 1959 demonstrated the hypocholesterolemic effects of intramuscularly administered androsterone (1). Because of our previous interest in the interrelationships of sex hormones, lipid metabolism and atherosclerosis, we undertook a more extensive investigation of this interesting phenomenon.

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In the initial studies, androsterone was administered orally because it had been shown by Dorfman and Hamilton in 1940 that this compound was efficiently absorbed by way of the gastro-intestinal tract (2). Thus, if similar physiologic effects could be obtained, patients could be spared the ordeal of daily intramuscular injections. Unfortunately androsterone orally, in the dosages used in this first study, had virtually no effect on the level of serum lipids. Therefore, the present studies include data on the intramuscular administration of androsterone. Moreover, the investigation covers not only the various serum lipid parameters but also the urinary steroidal metabolites.

MATERIALS AND METHODS

Patients

Thirteen middle-aged men with coronary heart disease were chosen for a trial of androsterone, administered orally as 50-mg. capsules twice daily for thirty days. All but one of these patients had grossly abnormal serum lipid levels. Eight male subjects volunteered for the parenteral studies. Six of these men had coronary heart disease, 1 had questionable angina pectoris, and 1 was a young clinically normal resident surgeon with an abnormal level of serum lipids. Six subjects received intramuscularly 50 mg. daily of androsterone in a 15 per cent benzyl alcohol-sesame oil vehicle (1). The other 2 were given a daily injection of a similar dose in the form of a microcrystalline aqueous suspension containing 0.5 per cent benzyl alcohol.

All subjects were checked frequently for any signs or symptoms of androgenic or anabolic activity attributable to androsterone, as well as any possible deleterious effects on cardiac function. Special attention was paid to changes in body weight or libido, and to local or systemic drug reactions.

Biochemical methods

Serum lipids. Total serum cholesterol and phospholipids (3), and ultracentrifugally separated "α-" and β-lipoprotein cholesterol levels (4) were determined by the standard methods of this laboratory. Serum triglycerides were estimated by the chloroform-soluble, alkali-hydrolyzed glycerol method of Van Handel and Zilversmit (5) after validation by interlaboratory comparison against the titrimetric method of Albrink[1] (6). The results are shown in Table 1. Twenty-two sera were cross-checked. The average value by the titrimetric method was 17 per cent higher than by the glycerol method, the 95 per cent confidence limits being ± 13 per cent. When the sera with very high triglyceride levels were excluded, the agreement was slightly better, the mean difference being 13.5 per cent with ± 10.6 per cent confidence limits.

Urinary ketosteroids. Complete 48-hour urine collections were frozen at $-20°$ C. until analyzed. The differential hydrolysis method of Lieberman, Mond and Smyles (7) was used, except that in specimens obtained during androsterone administration, two portions of commercial mammalian β-glucuronidase (Ketodase®, Warner-Chilcott) were added during the four-day incubation period to assure complete hydrolysis of the large amounts of androsterone glucuronide excreted. Gradient elution chromatography (8)

[1] Serum samples were kindly provided by Margaret J. Albrink, M.D., Department of Medicine, Yale University School of Medicine, New Haven, Connecticut.

TABLE 1. INTERLABORATORY COMPARISON OF SERUM TRIGLYCERIDE METHODS

No. of serum samples	Mean serum triglyceride levels (mg./100 ml.)			95% confidence limits
	Albrink (titrimetric)	Van Handel and Z. (glycerol)	Difference	
22	535	456	79	±57
14*	193	170	23	±18

* Samples containing more than 350 mg. per 100 ml. by the titrimetric method were excluded.

was employed to isolate the liberated ketosteroids, and identification was made by means of infrared spectroscopy in carbon-disulfide solution against authentic steroid spectra [as recorded in our library or published collections (9)]. Serum protein-bound iodine and total iodine determinations[2] were made by a modification of the method of Zak *et al.* (10).

RESULTS

The 13 patients who received androsterone orally for one month experienced few, if any, clinical effects. None reported any change in libido. Two claimed an increased sense of well-being. Six gained 0.5 to 1.4 Kg., 2 lost 0.5

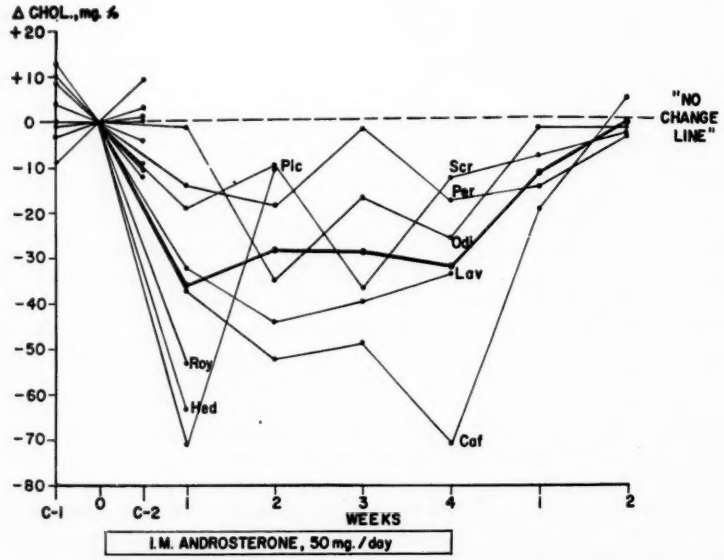

FIG. 1. Decrease of total serum cholesterol concentration during intramuscular administration of androsterone.

[2] Performed at the Boston Medical Laboratory, Boston, Massachusetts—Joseph and Norbert Benotti, Directors.

Kg., and 5 showed no change in body weight; the average weight gain was 0.3 Kg. Intramuscular administration, however, caused mild to severe local reactions in 7 of the 8 men studied. These consisted of varying degrees of pain, swelling, heat, and redness at the sites of injection. Mild to moderate systemic reactions occurred in 4 subjects, consisting primarily of low-grade fever, malaise, and occasionally muscle and joint pains.

The effects of both oral and intramuscular administration of androsterone on the levels of serum lipids are shown in Table 2. For comparison, the lipid changes in a group of men receiving a small daily oral dose of a po-

TABLE 2. CHANGES FROM CONTROL LEVELS INDUCED BY ANDROSTERONE ORALLY AND INTRAMUSCULARLY AND BY METHYLTESTOSTERONE ORALLY

Androgen, and route of administration	No. of subjects	Changes in cholesterol and phospholipids			Changes in α- and β-cholesterol			Change in triglycerides (mg./100 ml.)
		Cholesterol (mg./100 ml.)	Phospholipids (mg./100 ml.)	C/P ratio	$\Delta\alpha$-Cholesterol (mg./100 ml.)	$\Delta\beta$-Cholesterol (mg./100 ml.)	$\Delta\beta$-/α-ratio (%)	
Androsterone *orally* 100 mg./d. for 1 mo.	13	−2	−4	+0.02	−3	− 7	+ 7	—
Androsterone *intramuscularly* 50 mg./d. for 1 wk.	8	−36±9.0*	−24±5.5*	−0.02	−2	−34±8.3*	− 9†	−28±5.6*
Methyltestosterone *orally* 10 mg./d. for 3 mos.	12	+11	0	+0.02	−4	+11	+30*	—

* $P \leq 0.01$.
† $0.01 < P \leq 0.05$.

tent androgen, 17α-methyltestosterone, are also listed. Androsterone orally, after one month of dosage with 100 mg. daily, had no significant influence on any of the lipid parameters studied. But there was a suggestion of an increase in the β-/α-lipoprotein cholesterol ratio, as seen during oral administration of methyltestosterone. In contrast, intramuscular administration of androsterone in a dosage of 50 mg. daily for only one week produced in 8 subjects highly significant decreases in the levels of total serum cholesterol, phospholipids, and triglycerides. Ultracentrifugal flotation revealed that almost all of the decrease in serum cholesterol concentration was accounted for by a decrease in the β-lipoprotein fraction. Since there was little change in α-lipoprotein cholesterol, the derived β-/α-ratio also decreased significantly.

In Figure 1 are shown the individual decreases of total serum cholesterol concentration during one to four weeks of intramuscular androsterone administration. These changes are referred to the individual mean control values as zero, and the variation between two pretreatment control determinations is also shown. Termination of therapy is indicated by the first three letters of each patient's surname. The mean of these changes is represented by the heavy line. The average decrease of serum cholesterol was

quite constant throughout the four-week period of treatment and, in the 4 subjects on whom post-control determinations were made, there was a rise toward pre-treatment levels. In Figure 2, the individual decreases of β-lipoprotein cholesterol are presented in a similar way. These changes paralleled those of total serum cholesterol. The changes in serum triglyceride levels are shown in Figure 3. With the exception of Patient Scr., who was given microcrystalline androsterone in aqueous suspension, all subjects showed a

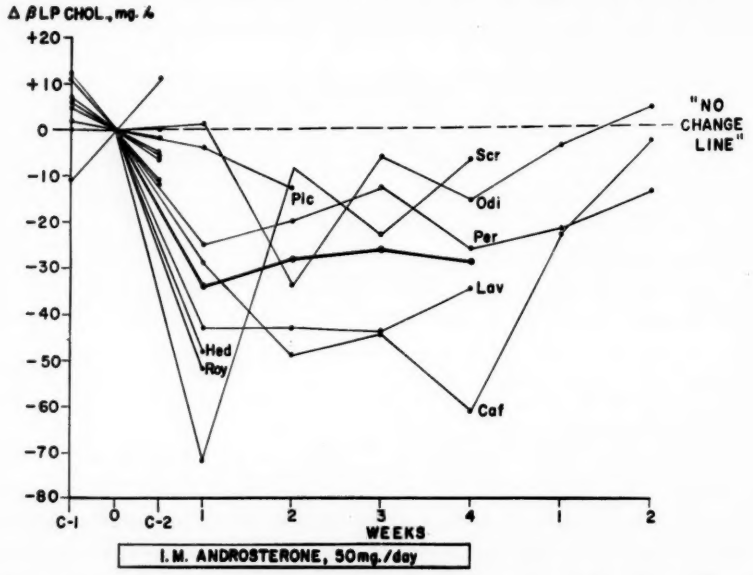

FIG. 2. Decrease in the level of serum β-lipoprotein cholesterol during intramuscular administration of androsterone.

consistent fall in the level of triglycerides during therapy, including Patient Pic. who also received the microcrystalline material.

In Figure 4 are shown the changes in the individual urinary 17-ketosteroids during oral and intramuscular administration of androsterone. The percentage value at the top of each bar is the fraction of the daily dose recovered in the urine. That there was good absorption of androsterone from the gastro-intestinal tract is indicated by the recovery in the urine of 40 and 42 per cent of the oral dose. In a single subject, free, glucuronide, sulfate, and residual hot acid-released steroids were chromatographed separately. Of the total androsterone, 93 per cent was recovered in the glucuronide fraction, and the remainder in the sulfate fraction. In general, recoveries were higher after intramuscular administration in benzyl alcohol-sesame oil. However, only 28 per cent of the steroid was recovered

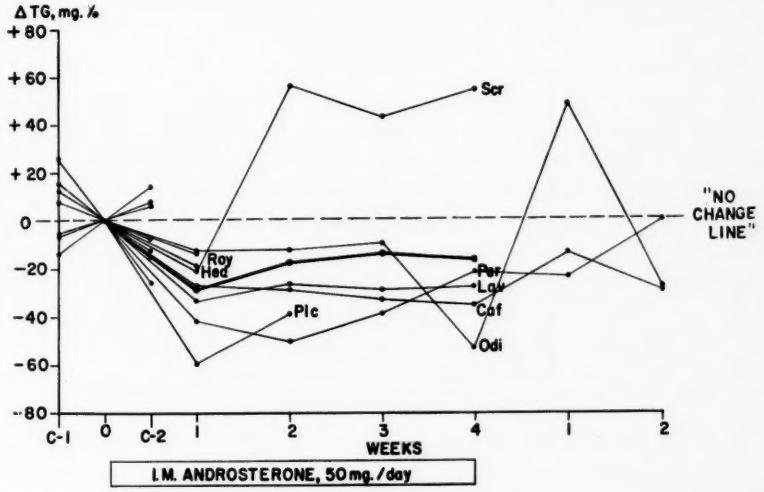

FIG. 3. Change in level of serum triglycerides during intramuscular administration of androsterone.

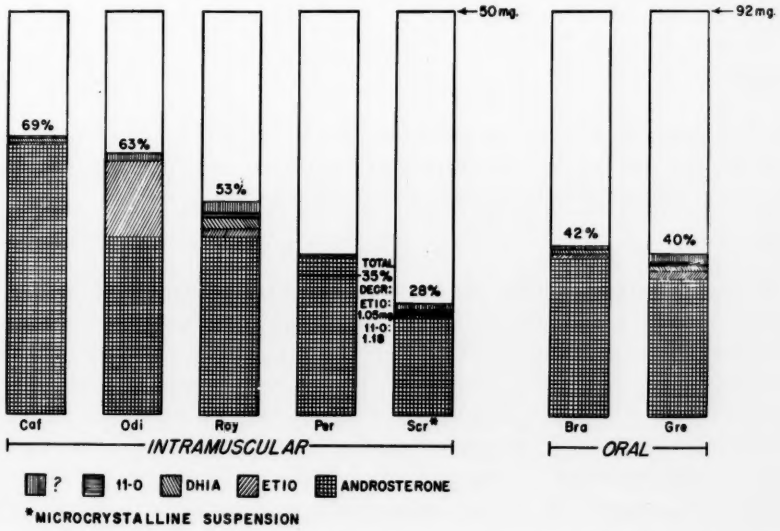

FIG. 4. Increased excretion of urinary 17-ketosteroids during administration of androsterone. "11–0" = sum of androstan-3α-ol-11,17-dione, androstan-3α,11β-diol-17-one, etiocholan-3α-ol-11,17-dione, and etiocholan-3α,11β-diol-17-one; "DHIA" = dehydro*iso*androsterone plus *iso*androsterone; "ETIO" = etiocholan-3α-ol-17-one; "?" = unidentified.

in one subject who received the microcrystalline suspension. Androsterone *per se* represented the major fraction of the total increase in 17-ketosteroid excretion during treatment, except in one subject (Odi.) in whom only 44 per cent was recovered as androsterone and 20 per cent as etiocholanolone. This apparently reflected a secular variation of endogenous etiocholanolone production, rather than a specific 5α- to 5β-transformation.

*Iso*androsterone (androstan-3β-ol-17-one) was sought in 6 pretreatment urine specimens by quantitative infrared spectroscopic examination of dehydro*iso*androsterone (Δ^5-androsten-3β-ol-17-one) fractions after chromatography (Table 3). It was found in 4 such specimens, in amounts

TABLE 3. URINARY EXCRETION OF *iso*ANDROSTERONE BEFORE AND DURING ADMINISTRATION OF ANDROSTERONE

(Patient BRA. received 92 mg. per day orally; the others received 50 mg. per day intramuscularly.)

Patient	*Iso*androsterone excretion (mg./24 hrs.)		% Recovery
	Control	Androsterone	
BRA.	Nil	0.40	0.43
ODI.	Nil	1.31	2.60
SCR.	0.15	0.62	0.94
ROY.	0.14	0.76	1.24
PER.	0.05	0.23	0.36
CAF.	0.05	0.45	0.80
		Mean % recovery	1.06

ranging from 0.05 to 0.15 mg. per twenty-four hours. During androsterone therapy, the amount of *iso*androsterone increased in all urines, the excess averaging 1.06 per cent of the dose (range, 0.36 to 2.60 per cent).

DISCUSSION

A possible explanation for the hypocholesterolemic effect of androsterone administered intramuscularly is the conversion of a small fraction of the androsterone to an estrogen. That this is not the case is suggested by the fact that serum phospholipid levels decreased and α-lipoprotein cholesterol levels remained unchanged during intramuscular androsterone therapy. whereas during estrogen therapy there was a marked increase in the level of both phospholipids and α-lipoprotein cholesterol. However, the observed lipid effects of androsterone intramuscularly were indeed "thyromimetic," in that not only did the concentration of serum cholesterol decrease, but that of phospholipids fell to a lesser extent, while the lipoprotein effect was primarily a decrease in the β-fraction. Similar effects of L-triiodothyronine (11) and L-thyroxine (12) have been reported by Oliver and Boyd. In order to be considered "thyromimetic" in any meaningful sense, a compound

should be capable of replacing thyroid hormone in the maintenance of myxedematous patients, or at least of significantly increasing oxygen consumption. Although Hellman and colleagues (1) stated that "one myxedematous patient showed an increase in basal oxygen consumption during treatment with androsterone" (from 85 ±7 to 99 ±6 ml./sq. m./minute), this does not represent adequate evidence for such an effect. As a physiologically active thyromimetic agent, androsterone might be expected to be involved in feedback control over thyroid function, presumably by inhibition of pituitary thyrotropin, leading to decreased synthesis of thyroxine and thereby a lowered level of serum protein-bound iodine. To test this hypothesis, sera which had been obtained before and during intramuscular administration of androsterone in 7 subjects, were code-numbered and submitted to an independent laboratory for determination of protein-bound iodine. The results are shown in Table 4. No significant changes occurred in the 6 subjects for whom satisfactory samples were available. L-thyroxine given in amounts sufficient not only to increase oxygen consumption but also to lower the serum cholesterol level to the observed values would have been expected to increase the severity of the attacks of angina pectoris in the 6 coronary patients of the present study (12). That this did not occur is additional evidence that the lipid effects of androsterone are not explicable by its thyroxine-like action.

TABLE 4. EFFECT OF INTRAMUSCULAR ADMINISTRATION OF ANDROSTERONE ON SERUM PROTEIN-BOUND IODINE LEVEL

Patient	Serum PBI (μg./100 ml.)	
	Control	After androsterone i.m.
CAF.	5.4	4.8
SCR.	5.8	6.0
LAV.	5.4	—*
ODI.	5.2	5.8
ROY.	4.6	4.2
PER.	5.2	4.6
HED.	5.8	5.8
Mean	5.2	5.2

* Sample contaminated; PBI and total iodine both greater than 20 μg. per 100 ml.

Since most of the subjects experienced local or systemic reactions, or both, it is clear that intramuscular androsterone is unsuitable for clinical use as an hypocholesterolemic agent. The explanation for such systemic reactions is not apparent. "Etiocholanolone fever" (13) was ruled out by demonstrating that there was no detectable contamination of the androsterone by this steroid. Although 0.7 per cent of *iso*androsterone was present in the administered material, earlier preliminary studies by Kappas *et al.*

indicated that both this steroid and androsterone itself are not pyrogenic (13). A minor additional component was isolated, which had an infrared spectrum suggesting a steroid polyhydroxy compound, but insufficient material was available for identification. Whether this contaminant might have been a steroid of the pregnane or etiocholane (5β) series, known to cause systemic reactions (14, 15), is not yet known. Although the small amounts of *iso*androsterone regularly found in urine during administration of androsterone may have in part arisen from the contaminating *iso*androsterone, more was isolated than could be accounted for in the material administered. Therefore *iso*androsterone was, in fact, a metabolite, as demonstrated by Schneider and Lewbart after oral administration of androsterone (16).

Acknowledgments

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JCEM

STEROID BIOSYNTHESIS IN THE HUMAN OVARY*†

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ABSTRACT

Steroid biosynthesis by normal and abnormal ovarian tissue was studied *in vitro*. From the incubation media, 6 substances were isolated; 5 of these were characterized as unchanged progesterone, 17α-hydroxyprogesterone, androstenedione, 20α-hydroxy-4-pregnene-3-one and 17α,20α-dihydroxy-4-pregnene-3-one. Correlation of synthetic pattern and production rates with morphology revealed that follicular and thecal tissues were active in 17-hydroxylation and that capsular, stromal and connective tissue were active in 20-reduction. Specimens from Stein-Leventhal ovaries and other luteinized structures yielded the largest amounts of 17α-hydroxyprogesterone. Increased quantities of androstenedione were produced by the luteinized portions of the Stein-Leventhal ovaries. Medullary islets of theca luteal cells were not impressively active in either 17-hydroxylation or side-chain cleavage to androstenedione. It is concluded that the cystic structures of the Stein-Leventhal ovary have the capacity to synthesize greater amounts of recognized androgenic compounds than do normal ovaries.

IN A subsequent publication (1) we shall present clinical evidence that polycystic ovarian disease results from an ovarian lesion correlated with biosynthetic alterations in the ovary, and is cured by surgical resection of the ovary. Although plausible pathways of steroid synthesis by gonadal tissue have been reported (2–4), correlation of synthetic capacity with ovarian morphology and clinical status is not well established.

This study was undertaken to determine the quantitative amounts of various steroids synthesized *in vitro* from 4-C^{14}-progesterone substrate by normal and abnormal ovarian tissue.

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MATERIAL AND METHODS

Incubation of ovaries

After representative portions were taken for microscopic examination, fresh tissue was frozen and incubated within seventy-two hours, using a modification of the system reported by Hayano *et al.* (5). The tissue was minced and added in multiples of 1.0 Gm. to the incubation medium which contained:

a) Phosphate buffer	20 mM, pH 7.4 (10 ml. per Gm. of ovary)
b) Sodium fumarate	70 mM
c) Nicotinamide	40 mM
d) Magnesium chloride	7 mM
e) Adenosine triphosphate as sodium salt (ATP)	4 mM
f) Diphosphopyridine nucleotide (DPN)	0.4 mM
g) Tryphosphopyridine nucleotide (TPN)	0.4 mM
h) Progesterone 4-C^{14}, repurified by chromatography	

In incubations 4a, 4b, 4L and 7 (see Table 2), 18 μg. of progesterone with a specific activity of 55 μC per mg. was used per Gm. of tissue. In all other incubations, 40 μg. (specific activity 5 μC per mg.) was used per Gm. of tissue. The flask was flushed with 95 per cent oxygen and 5 per cent carbon dioxide, and incubated for four hours.

Extraction and fractionation

Solvents were purified in accordance with standard techniques (6) and redistilled prior to use. Evaporation of volumes exceeding 10 ml. was effected by drying in vacuum; lesser volumes were evaporated under a fine stream of filtered dry air. Carrier androstenedione was added in all cases after the incubation. Synthesis was terminated by precipitation of protein with 80 per cent acetone. The precipitate was removed by filtration and the acetone removed in partial vacuum. The aqueous residue was extracted three times with 2 volumes of chloroform, taken to dryness and the residue partitioned between Skellysolve B and 70 per cent ethanol. After removal of the ethanol, the aqueous fraction was re-extracted with chloroform, and then taken to dryness. The steroid residue was partitioned between ether and 1 *N* sodium hydroxide, and the pH of the aqueous portion was adjusted to 7. The aqueous fraction, containing the phenolic steroids, was then re-extracted with ether. The neutral and phenolic fractions were taken to dryness for chromatography.

Chromatography and determination of radioactivity

All chromatography was carried out on Whatman #2 paper utilizing systems described by Zaffaroni (7). A Nuclear-Chicago manual windowless gas-flow counter was used both for scanning chromatograms and for counting the radioactivity of the eluted steroids. The latter were plated on tared aluminum planchets at infinite thinness, and sufficient counts were made to give a standard error of not more than 2 per cent.

Preparation of derivatives

1. *Acetylation.* To the steroid to be acetylated, a few drops of pyridine and acetic anhydride were added in a ratio of 1:2. After sitting overnight at room temperature, the pyridine and acetic anhydride were removed by drying in vacuum.

2. *Reduction.* A modification of the method of Norymberski and Woods (8) was used. The steroid was added to 2 ml. of anhydrous methanol containing 10 mg. of sodium

borohydride. After ninety minutes at 0° C., a drop of glacial acetic acid was added, the methanol removed by vacuum, water added, and the steroid extracted with ethyl acetate.

3. *Oxidation.* A few crystals of chromium trioxide were washed with glacial acetic acid, dried in vacuum and added to the steroid. Ten drops of glacial acetic acid were added and the mixture was allowed to react overnight. Water was then added and the steroid extracted with ethyl acetate.

4. *Hydrolysis of steroid acetates.* The steroid acetate was incubated with 12 units of porcine renal acylase (Armour) in phosphate buffer, pH 7.2, at 37° C., for four hours and extracted with ethyl acetate. Previous experience in this laboratory has demonstrated that this will quantitatively hydrolyze most primary and secondary steroid alcohols.

5. *Periodic-acid oxidation* (9). One volume of glacial acetic acid and 5 volumes of 0.4 *N* sulfuric acid containing 0.06 *M* periodic acid were added to the steroid. After three hours, distilled water was added and the steroid extracted with ethyl acetate.

Proof of structure

After isolation of individual steroids, specific activities of progesterone and 17-α-hydroxyprogesterone were determined when possible. Endogenous steroids did not cause isotopic dilutions exceeding 25 per cent, except in incubation 4L (persistent corpus luteum) in which a tenfold dilution was seen. After purification and partial characterization, similar fractions from all incubations were combined for final proof of structure by the carrier technique of Berliner and Salhanick (10). In no case did subsequent data reveal that the combinations contained other than the anticipated pure steroids.

RESULTS

Substances isolated (Table 1)

The phenolic portion contained less than 1 per cent of the radioactivity recovered from all incubations. On chromatography only small peaks in-

TABLE 1. PROOF OF STRUCTURE OF STEROID PRODUCTS*

Steroid	Reaction	Rf identical with that of	System	Specific activity (cpm/μM)
17α-Hydroxyprogesterone	———	17α-hydroxyprogesterone	hexane (22 hrs.)	12,550
	chromate oxidation	androstenedione	hexane:benzene	13,600
	reduction	testosterone	hexane:benzene	13,900
Androstenedione	———	androstenedione	hexane:benzene	7,620
	reduction	testosterone	hexane:benzene	7,000
	acetylation	testosterone acetate	hexane	7,570
20α-Hydroxy-4-pregnene-3-one	———	20α-hydroxy-4-pregnene-3-one acetate	hexane:benzene	16,700
	hydrolysis	20α-hydroxy-pregene-4-one	hexane:benzene	15,800
	chromate oxidation	progesterone	hexane	15,500
17α:20α-Dihydroxy-4-pregnene-3-one	———	17α:20α-dihydroxy-4-pregnene-3-one	hexane:benzene (22 hrs.)	3,820
	periodic acid	androstenedione	hexane:benzene	4,020
	reduction	testosterone	hexane:benzene	3,640

* Authentic steroid was added after isolation of individual products. Specific activity was determined before and after the formation of each consecutive derivative. Amounts of steroids were determined by optical density at 240 mμ.

consistently displaying the Rf's of standard estrone and estradiol were found.

Though many apparent components were separated from the neutral fraction by chromatography, most of the radioactivity was found in one of 6 major fractions. Five of these were purified and characterized as follows:

Unchanged progesterone. This steroid displayed maximal absorption at 240 mμ, had the Rf of standard progesterone in the hexane-formamide system, and resisted acetylation.

17α-Hydroxyprogesterone. This steroid also had an absorption maximum at 240 mμ, as well as the Rf of standard 17α-hydroxyprogesterone in hexane:formamide (twenty-two hours) and hexane:benzene-formamide systems. It resisted acetylation. After addition of carrier, chromic-acid oxidation yielded a derivative with the Rf of androstenedione, and subsequent reduction yielded a substance with the Rf of testosterone. Specific activity of the derivatives remained constant (Table 1).

4-Androstene-3:17-dione. This steroid demonstrated the Rf of standard androstenedione in hexane:formamide (twenty-two hours) and hexane:benzene-formamide systems. It resisted acetylation. After carrier was added, reduction yielded a compound with the Rf of testosterone and subsequent acetylation led to a compound with the Rf of testosterone acetate. Specific activity remained constant (Table 1).

20α-Hydroxy-4-pregnene-3-one. This steroid displayed the Rf of standard 20α-hydroxy-4-pregnene-3-one in the hexane-formamide system (twenty-two hours). On acetylation, a compound with the Rf of the standard acetate in the same system was formed. Carrier was then added. Hydrolysis yielded a compound with the Rf of 20α-hydroxy-4-pregnene-3-one, and subsequent oxidation one with the Rf of progesterone. Specific activity remained constant (Table 1).

"Origin fraction." This fraction remained at the origin in the hexane:formamide system (twenty-two hours). It moved like a single compound in the hexane:benzene-formamide (Rf, .07), benzene-formamide (Rf, .30) and benzene:chloroform-formamide (Rf, .42) systems. An absorption maximum was present at 240 mμ. This fraction was combined from all but three of the incubations. Re-chromatography in the hexane:benzene-formamide system for twenty-two hours yielded two distinct components, designated as O_1 and O_2 in order of decreasing polarity.

17α:20α-Dihydroxy-4-pregnene-3-one. Fraction O_2 contained approximately one-fifth of the total counts in the origin fraction. Gravimetrically, the amount present was insufficient to permit unequivocal spectroscopic analysis. Periodic-acid oxidation caused formation of a compound that had the Rf of standard androstenedione in the hexane-benzene:formamide system and resisted acetylation. Another aliquot of the unknown was

chromatographed with 17α:20α-dihydroxy-4-pregnene-3-one and its 20β epimer as standards. The Rf corresponded to that of the 20α epimer. Therefore, carrier 17α:20α-dihydroxy-4-pregnene-3-one was added to the radioactive steroid. Periodic-acid oxidation again resulted in a compound with the Rf of androstenedione in the hexane:benzene-formamide system and subsequent reduction formed a compound with the Rf of testosterone in the same system. Specific activity remained constant (Table 1).

Fraction O_1 demonstrated maximal light absorption at 240 mμ and was unaltered by periodic-acid oxidation. Present effort is being directed toward identification, characterization and bioassay of this substance (11).

Correlation of morphology with synthetic pattern

Complete case histories, laboratory data and pathologic descriptions will be published elsewhere (1). Tissues from 9 patients were studied. From a

TABLE 2. RECOVERY OF STEROID AFTER INCUBATION WITH 4-C^{14}-PROGESTERONE*

Steroid	"Normal"			Persistent C.L.			Stein-Leventhal syndrome					Postmenopausal
	1	2	3	4a	4b	4L	5m	5c	6	7	8	9
Origin	10	9	5	5	4	12	10	9	17	19	12	—
17α-Hydroxyprogesterone	20	20	6	6	6	17	7	18	18	37	23	—
Androstenedione	1.6	1.4	0.7	0.6	0.7	1.5	1	3	8	6	4.2	—
20α-Hydroxy-4-pregnene-3-one	5	8	8	8	13	1	6	4	5	4	2	5
Progesterone	35	29	34	40	36	25	30	35	2	4	14	79

* All data are in terms of per cent of starting material. The yields are convertible to μg. steroid/Gm. tissue/hour because the incubations contained proportional amounts of all factors. The origin material consisted of about 20 per cent 17α:20α-dihydroxy-4-pregnene-3-one and 80 per cent of an as yet uncharacterized substance (13).

Abbreviations: C.L. = corpus luetum; 4a = capsular tissue; 4b = capsular and medullary tissue; 4L = persistent corpus luteum tissue; 5m = medullary tissue; 5c = cortical tissue.

patient with a persistent corpus luteum, 3 types of tissues were incubated (4a, 4b and 4L); and from a patient with the Stein-Leventhal syndrome, 2 types of tissue were studied (5m and 5c representing cortex and medulla, respectively). The data are listed in Table 2. Representative histologic sections are presented in Figure 1.

After formalin fixation and staining with hematoxylin and eosin, the ovaries were divided histologically into the following groups (Figs. 1 & 2):

1. *Normal group* (specimens 1, 2 and 3; the incubated portions including both cortex and medulla): *a*) Control specimens were taken in the proliferative phase (day 8 and day 9) from patients aged 31 and 33 years. The ovaries were normal in size and both contained numerous pre-antral follicles and several small cystic follicles (2–4 mm. in diameter) with minimal luteinization of the theca interna. These tissues were very similar in biosynthetic pattern. Approximately one-third of the radioactivity was recovered as unchanged progesterone, 20 per cent as 17α-hydroxyprogesterone, 10 per cent as "origin" fraction, 5 per cent and 8 per cent respec-

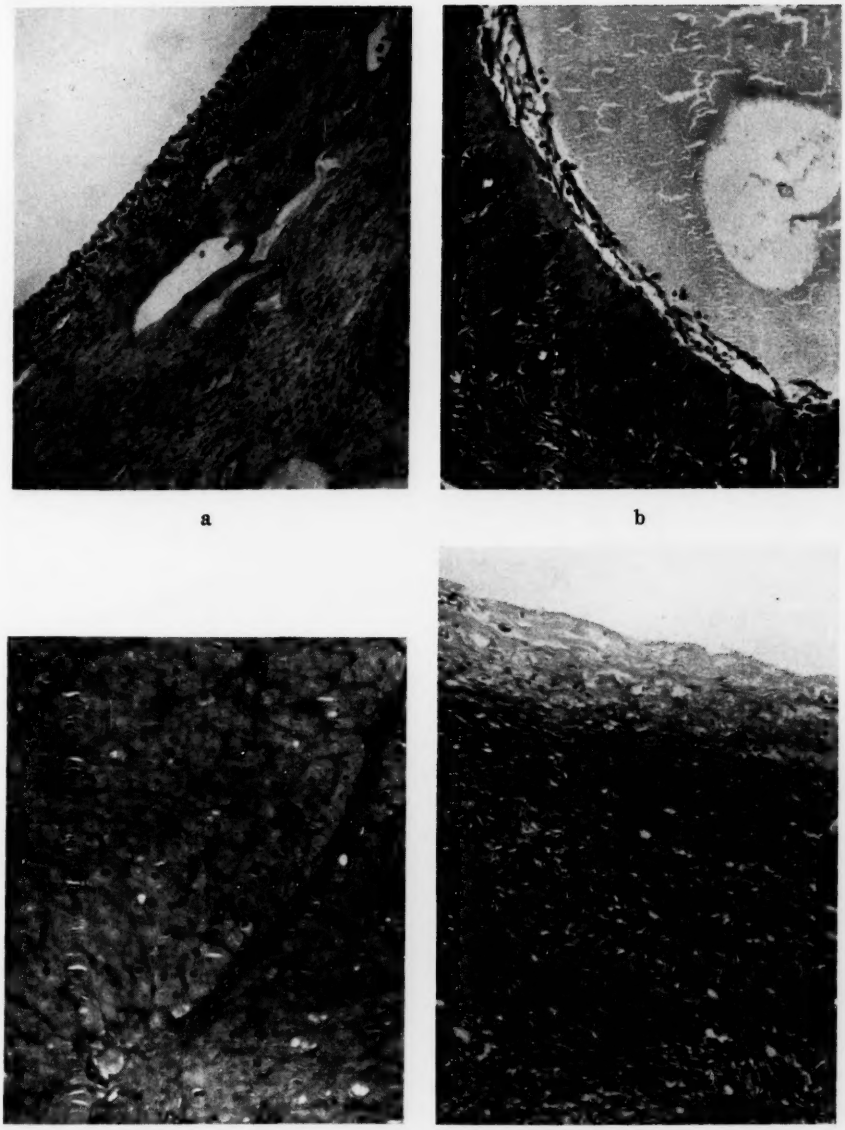

FIG. 1. Microscopic sections of ovaries utilized in incubations:

a. Control ovary (specimen 2) showing wall of antral cyst. Note absence of developed theca.

b. Ovary containing persistent corpus luteum (specimen 4a) showing wall of small cyst. Note absence of granulosa and theca.

c. Corpus luteum structure from same ovary (specimen 4L).

d. Atrophic ovary (specimen 9). Note absence of any follicular structures.
(Reduced from ×140.)

tively as 20α-hydroxy-4-pregnene-3-one, and about 1.5 per cent as androstenedione. *b*) Specimen 3 was a grossly normal ovary taken from a 41-year old patient approximately twelve days after ovulation. (The corpus luteum was in the other ovary.) Though it contained many small follicular structures, no cystic follicles were present. It yielded only 6 per cent 17α-hydroxyprogesterone, 5 per cent origin fraction, 8 per cent 20α-hydroxy-4-pregnene-3-one, and 0.7 per cent androstenedione.

2. *Persistent corpus luteum*: Specimens 4a, 4b and 4L were removed from a 22-year-old girl with amenorrhea. The enlarged right ovary contained a corpus luteum persistans and the left ovary was slightly enlarged. Both contained pre-antral follicles and numerous small cysts without granulosal or thecal elements. Specimen 4a consisted of the capsular and subcapsular region of both ovaries. Specimen 4b consisted of these areas as well as medulla containing fibrous tissue but without follicular elements or hilar cells. Both specimens yielded steroids in proportions similar to those for specimen 3, with the exception that 18 per cent 20α-hydroxy-4-pregnene-3-one was present in 4b.

Specimen 4L was the luteinized structure contained in the grossly enlarged right ovary. It had been seen with a culdoscope eighteen weeks previously. Although histologically normal and noncystic, it measured 2 cm. in diameter. This luteinized tissue displayed a synthetic pattern somewhat like that of specimens 1 and 2 which showed little or no luteinization, yet there was a tenfold dilution of specific activity by endogenous steroids. It yielded 17 per cent 17α-hydroxyprogesterone, 12 per cent origin fraction, 1.5 per cent androstenedione, but only 1 per cent 20α-hydroxy-4-pregnene-3-one. Little or no fibrous tissue or medulla was present.

3. *Luteinized polycystic ovaries (Stein-Leventhal syndrome)*: These ovaries were grossly enlarged, and demonstrated luteinized polycystic changes as classically described in the Stein-Leventhal syndrome. Two of the specimens were generously provided by Dr. Michael Leventhal. Each of these patients had secondary amenorrhea with hirsutism, and all responded to wedge resection by return of normal menses.

Though Patient 5 was very hirsute, the wedge biopsy specimens were less striking in respect to luteinized cysts but contained numerous islets of foamy epithelioid theca-lutein cells in the medulla. The cortex and medulla were incubated separately. In synthetic pattern, the medulla (specimen 5m), which contained the theca-lutein islets, was similar to specimens 3, 4a and 4b and yielded 7 per cent 17α-hydroxyprogesterone, 10 per cent origin fraction, 1 per cent androstenedione and 6 per cent 20α-hydroxy-4-pregnene-3-one. The cortex (5c) synthesized 18 per cent 17α-hydroxyprogesterone and 3 per cent androstenedione.

The other ovaries associated with the clinical and morphologic criteria of

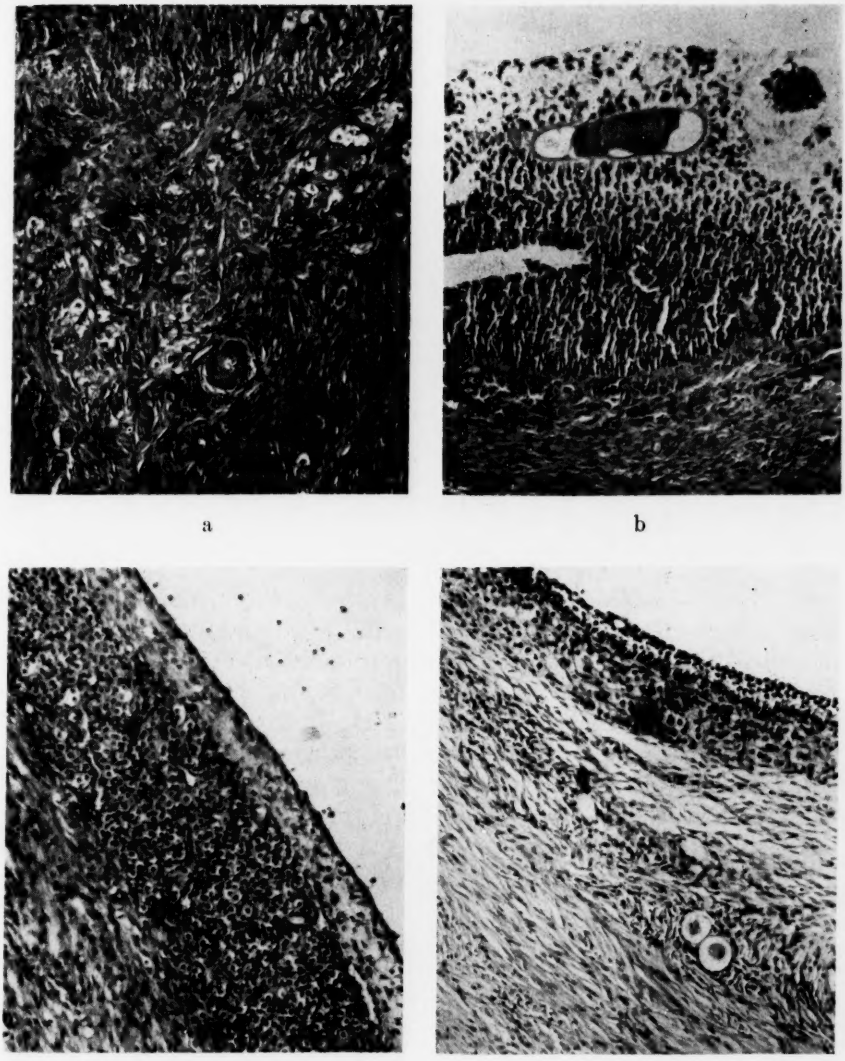

FIG. 2. Microscopic sections of ovaries used in incubations:

a. Medulla of polycystic ovary (specimen 5m). Note islets of foamy theca cells. These were found throughout the medulla.

b. Cyst wall from cortical region of the same ovary (specimen 5c) displaying atretic ovum and moderate theca.

c. Cyst wall from polycystic ovary (specimen 7). A wide layer of luteinized theca cells remains after disappearance of granulosa.

d. Cyst wall from polycystic ovary (specimen 8). Luteinized theca cells are less prominent than in specimen 7.

(Reduced from ×140.)

the Stein-Leventhal syndrome exhibited the highest conversions of all ovarian tissue studied except corpus luteum. They yielded proportions of 17α-hydroxyprogesterone ranging from 17 to 37 per cent; origin fraction from 12 to 19 per cent; and androstenedione, 8 per cent, 6 per cent, and 4.2 per cent respectively. Almost all of the progesterone was converted.

4. *Atrophic ovary:* Specimen 9 was taken from an 81-year-old woman, 32 years postmenopausal. It was capable of reducing the substrate to 20α-hydroxy-4-pregnene-3-one in amounts only slightly less than did the other specimens, but it yielded no other steroids in any significant amount.

The patterns of the recovered steroids are shown in Table 2. In all incubations, carrier androstenedione was added to the incubation medium before extraction, and corrections for losses of this steroid made. As shown with the carrier technique in later incubations, losses of other compounds approximating 30 per cent occurred fairly constantly. Corrections for these losses were not made.

DISCUSSION

Qualitative analysis of the steroids synthesized by gonadal tissues has been performed by several investigators and a few quantitative studies are available. Uniform results have not been observed, probably because of nonhomogeneity of the ovary and the different systems utilized. Often, the quantities of the steroids isolated were so small that critical proof of structure was impossible. We have attempted to alleviate these problems by correlating synthesis with histologic pattern and by reporting only those substances which could be adequately characterized. The addition of reduced cofactors, generating systems and gonadotropins was purposely avoided in order to evaluate the endogenous synthetic potential of the tissue.

The steroids isolated from these incubations conform to an accepted pathway (Fig. 3). Possible alternate pathways, however, should not be excluded. In general, when follicular or luteal tissue was present, reasonable conversion to 17α-hydroxyprogesterone and androstenedione occurred; in aliquots composed principally of connective tissue, 20α-hydroxy-4-pregnene-3-one was more prominent.

The absence of certain steroids is of interest. Testosterone was not present in demonstrable amounts in these incubations, nor was it isolated by Zander *et al.* from human ovarian tissue (12), by Short from the follicular fluid of the mare (13), or by Sweat *et al.* from human and bovine corpus luteum (4). Its presence has been reported after incubation of Stein-Leventhal ovaries with 1-C^{14}-acetate (14). Conversion of androstenedione to testosterone has been shown in testis (3) and ovary (15). Anliker *et al.* (16) and Savard *et al.* (17) were successful in isolating it from an arrhenoblas-

toma, but others were not [Wiest *et al.* 18]. There are, however, several histologic types of arrhenoblastomas. These differences may be due to differences in cofactor availability, or levels of the precursor, androstenedione, or 17-reductase activity of the tissues.

There was no evidence for the presence of 20β-hydroxy-4-pregnene-3-one, although it has been isolated by Lynn and Brown (3) from testicular, and by Sweat *et al.* (4) from ovarian incubations. Small amounts of substances displaying the Rf's of 17α:20β-dihydroxy-4-pregnene-3-one and

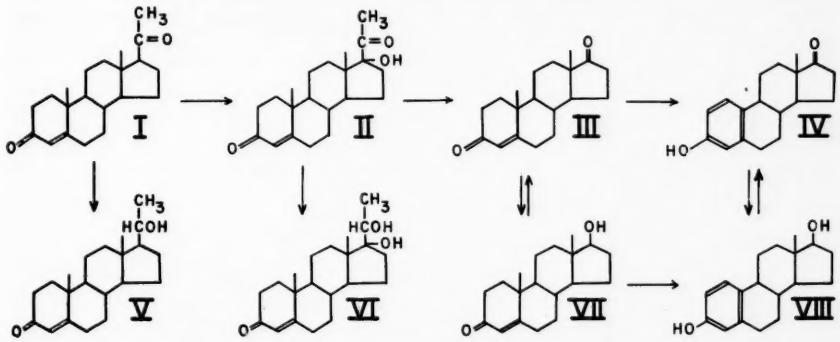

FIG. 3. Probable pathway of conversion of progesterone by human ovarian tissue. Substances: progesterone (I); 17α-hydroxyprogesterone (II); 4-androstene-3,17-dione (III); estrone (IV); 20α-hydroxy-4-pregnene-3-one (V); 17α:20α-dihydroxy-4-pregnene-3-one (VI); testosterone (VII); estradiol (VIII).

6β-hydroxyprogesterone were detected but these were not characterized. The former compound has been reported by Sandor and Lauthier (19) in their incubation studies with 17α-hydroxyprogesterone as substrate.

Marked changes in specific activity of the labeled steroid did not occur, indicating that only limited distortion of the product pattern can be ascribed to variation in the content of endogenous steroids. This allows simultaneous comparison of synthetic rates and patterns, both being indicated by the relative number of counts in the various identified fractions. The findings on persistent corpus luteum cannot be similarly compared, however, because a marked dilution of specific activity was noted.

The atrophic ovary was capable only of reducing progesterone to 20α-hydroxy-4-pregnene-3-one. The association of this compound with the presence of nonfollicular stroma in other incubations and its synthesis from progesterone by von Kuppfer cells (20) and uterine fibroblast cultures (21), as well as the data of Lynn and Brown (3), support its role as a degradation product rather than a precursor of another biologically active steroid.

Ovaries in the proliferative phase (day 8 and day 9) were more active in

synthesis than either specimens in the normal luteal phase (corpus luteum excluded) or specimens from the patient with the corpus luteum persistans (again "corpus luteum" excluded). This supports the suggestion of Allen *et al.* (22) that, during the luteal phase, areas of the ovary other than the corpus luteum itself are relatively quiescent, the latter structure alone maintaining steroid synthesis. The pattern of specimens 4a and 4b also shows poor synthetic capacity for the "functional" steroids in cysts devoid of follicular elements, but these cysts did synthesize considerable amounts of the 20α-hydroxy "degradation" product.

The polycystic luteinized specimens were most active synthetically, yielding much higher levels of androstenedione than did normal control tissue. Theca-cell islets have been considered both as structures accounting for hirsutism (23) and as degenerate cells. Our results with incubation of these tissues (5m) taken from a hirsute woman with enlarged polycystic ovaries and normal adrenal function appear to refute their role as cells involved in the production of recognized androgens in high quantities.

The corpus luteum persistans, on examination of synthetic pattern alone, appeared to be less active than polycystic ovaries. Nevertheless, when tenfold dilution by endogenous steroid is considered, total synthesis per gram of tissue was much higher. On the other hand, this tissue represented what appeared to be the entire active portion of the gonads in this patient (most of the luteinized structure was incubated), whereas relatively small proportions of the total mass of the enlarged polycystic ovaries were used. It is impossible to assess possible rates of progesterone synthesis in all incubations. Certainly, isotopic dilution in this manner would alter the apparent amounts of end-products. In most incubations it appears quantitatively unimportant; in the corpus luteum, obviously, it was significant.

The etiology of the clinical syndrome of amenorrhea, hirsutism and enlarged polycystic ovaries as described by Stein and Leventhal, has long been debated. Opinion based on changes in urinary steroid pattern and the return of menses after administration of cortisol has favored an adrenal origin, although biopsy specimens of the adrenal taken from women with this disorder have been reported to be normal (Leventhal, personal communication). More recently, Goldzieher and Axelrod (24) found that depression of urinary 17-ketosteroid excretion by exogenous corticosteroids was less in patients with the Stein-Leventhal syndrome than in normal controls. A good deal of confusion and contradiction exists in the literature, mainly because of lack of proper diagnoses by direct visualization and histologic study of the ovaries in the patients studied.

In addition to the steroids synthesized by the various tissues, the utilization of the substrate seems to be correlated with the amount of 17-hydroxylation. The Stein-Leventhal ovaries clearly utilized more of the added

progesterone, in contrast to the less active ovaries. It might be anticipated that the presence of large amounts of capsular and stromal connective tissue, as occurs often in polycystic ovaries, would lead to considerable formation of 20-hydroxylated derivatives. This was the case when Sandor and Lanthier used 17α-hydroxyprogesterone as a substrate, although the addition of human chorionic gonadotropin tended to increase the yield of androstenedione (19).

Androstenedione might accumulate in polycystic ovarian tissue incubations because of a genetic or other type of block in the conversion of androstenedione to estrogens. Certain facts, however, tend to refute this theory.

First, the syndrome is cured by resection of the ovary. Second, the presence of proliferative endometrium and adequate secondary sex development in the patient reflect the biologic action of estrogen. Moreover, the failure to demonstrate estrogen as an incubation product of a progesterone substrate is not reliable because the conditions may not have been optimal or adequate amounts of the immediate substrate may not have been available. It is pertinent that neither the normal ovarian tissue in the proliferative phase nor the corpus luteum tissue produced much estrogen.

Although it may be premature to state conclusively that an endocrine substance accounts for the hirsutism accompanying the Stein-Leventhal syndrome, the increased quantity of androstenedione, which is convertible by the ovary to testosterone, makes it an attractive postulate (15).

Acknowledgments

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THE INFLUENCE OF THYROID HORMONE ON HYDROCORTISONE PRODUCTION AND METABOLISM*

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ABSTRACT

The metabolism of endogenously produced and exogenous tracer doses of labeled hydrocortisone was studied in euthyroid subjects, in spontaneous hyperthyroidism, in myxedema, and following administration of triiodothyronine. It was found that an elevated level of thyroid hormone caused pronounced alteration in the metabolism of hydrocortisone toward 11-ketonic metabolites, together with an absolute increase in the endogenous production of the adrenal hormone. In hypothyroidism the converse of these effects was found, in that the metabolism of hydrocortisone was shifted toward the production of 11-hydroxy metabolites together with a decreased formation of hydrocortisone from endogenous sources. A mechanism is suggested for these changes in the production and metabolism of hydrocortisone.

THESE studies concern the effect of thyroid function on the metabolism of hydrocortisone in man. It was found that the level of thyroid hormone changed the total production of hydrocortisone by the human adrenal and, together with this, serious alterations in the peripheral metabolism of the hormone were evident. Spontaneous hyperthyroidism or the administration of triiodothyronine (T_3) caused a pronounced shift toward the formation of 11-ketonic metabolites accompanied by an absolute increase in hydrocortisone production as measured by its urinary metabolites[1] tetrahydrocortisone (THE), tetrahydrohydrocortisone (THF) and

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[1] The following trivial names and abbreviations are employed: Hydrocortisone for 11β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione. Cortisone for 17α,21-dihydroxy-Δ^4-pregnene-3,11,20-trione. Tetrahydrocortisone (THE) for 3α,17α,21-trihydroxypregnane-11,20-dione. Tetrahydrohydrocortisone (THF) for 3α,11β,17α,21-tetrahydroxypregnane-20-one. Allotetrahydrohydrocortisone (ATHF) for 3α,11β,17α,21-tetrahydroxyallopregnane-20-one. Cortols for a mixture of epimers at C-20 of pregnane-3α,11β,17α,20,21-pentols. Cortolones for a mixture of epimers at C-20 of 3α,17α,20,21-tetrahy-

allotetrahydrohydrocortisone (ATHF). The converse of these results was observed in hypothyroidism. The production of hydrocortisone was diminished in absolute amount and the alteration in metabolic transformation led to the preponderant production of 11-hydroxysteroids. Studies of the metabolic transformation of tracer doses of radioactive hydrocortisone confirmed these results. In myxedematous and euthyroid subjects, after administration of triiodothyronine, and in spontaneous hyperthyroidism, the tracer dose of hormone (with minor exceptions) was metabolized in the manner expected from the results with the endogenous steroids.

METHODS

Metabolites of endogenous hormones. The isolation, identification and quantitation of the various metabolites have been described in detail (1). In brief, metabolites with the dihydroxy acetone side-chain as well as the glycerol side-chain were isolated from the unfractionated neutral steroid extract obtained after enzymatic hydrolysis; 17-ketosteroids were isolated from a separate portion of the same extract after preparation of the ketonic fraction by treatment with Girard's reagent T. The individual reducing ketols were measured by the method of Weichselbaum, Margraf and Mack (2); 17-ketosteroids by a modified micro Zimmermann reaction (3); and cortols and cortolones by the method of Hurlock and Talalay (4).

Metabolites of exogenous hydrocortisone-4-C^{14}. The same methods of hydrolysis and separation employed for the endogenous metabolites were used with the metabolites of the labeled hormone. The quantitative analysis of these metabolites by paper chromatography has been described in detail (5). The 24-hour urine collection immediately following the tracer dose was employed for the analyses.

Patients. The pertinent clinical information, as well as a description of the individual studies, is shown in Table 1. The subjects are identified by initials, age and sex. The accuracy of all urine collections was checked by daily measurements of creatinine excretion. Many of the patients were hospitalized in James Ewing Hospital, whereas others were ambulatory. The 2 studies with adrenocorticotropic hormone (ACTH) were made at Montefiore Hospital. In certain instances it was possible to examine the same patient at different levels of thyroid function.

RESULTS

Studies of metabolism of endogenous hydrocortisone

Steroid values are expressed as mg. per day per Gm. of creatinine. The use of the creatinine correlation essentially compensates for differences in the size or net metabolic capacity of different subjects and permits comparison of the sexes on a common basis (6). Table 2 shows the daily production from endogenous sources of THE, THF and ATHF for a series of 10 control subjects, 6 of whom were again studied after treatment with triiodo-

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droxypregnane-11-one. 11β-Hydroxyetiocholanolone (OH-E) for 3α,11β-dihydroxyetiocholane-17-one. 11-Ketoetiocholanolone (11 = OE) for 3α-hydroxyetiocholane-11, 17-dione. Triiodothyronine (T_3) for 3,5,3′-triiodothyronine.

TABLE 1. CLINICAL STATUS AND TREATMENT OF PATIENTS

Subject, age & sex	Diagnosis	Creatinine excretion* (Gm./24 hrs.)	Treatment†
Euthyroid			
BS —28 F	Schizophrenia	0.9	T_3
BE —33 M	Schizophrenia	1.7	T_3
SR —23 M	Schizophrenia	1.5	T_3
WN—23 F	Schizophrenia	1.3	T_3
VN —24 M	Normal	1.6	T_3
SZ —24 M	Normal	1.5	T_3
ML—27 F	Normal	1.4	
FR —32 M	Normal	1.7	
GR —41 M	Normal	1.5	
MN—58 M	Multiple sclerosis	1.1	
Hyperthyroid			
KS —68 M	Graves' disease Cancer of prostate	0.9	I^{131} therapy
LB —61 F	Toxic nodular goiter	0.8	I^{131} therapy
ML—44 F	Graves' disease	1.0	I^{131} therapy
DO —43 M	Toxic nodular goiter	1.3	ACTH
GR —67 M	Toxic nodular goiter	1.2	I^{131} therapy
PK —46 F	Cancer of cervix		
Hypothyroid			
MA—42 F	Postoperative myxedema	1.2	Thyroid, 240 mg. per day for 2 mos.
SA —60 M	I^{131} myxedema Diabetes mellitus Congestive heart failure	1.4	
WE—59 F	Primary myxedema	0.6	
WR—68 M	Primary myxedema	1.0	
CR —72 F	Primary myxedema Diabetes mellitus Cancer of breast	1.2	
GZ —60 F	Myxedema Hashimoto's disease	0.5	
KA —34 F	Postoperative myxedema Malignant thymoma Toxic goiter (March 16, 1953)	1.0	
WY—65 F	Postoperative myxedema	1.1	ACTH
RS —66 M	Myxedema	0.9	
LN —77 F	Myxedema Cancer of breast Diabetes	0.9	

* In all subjects the daily creatinine excretion was constant within experimental error. Values are the average for the period of collection. When the same subject was examined at two different levels of thyroid hormone activity, these values remained the same in all cases except Subject VN who excreted 2.0 Gm./24 hours during T_3 therapy, and Subject KS who excreted 1.1 Gm./24 hours when he was euthyroid. The value for the specified collection was employed for the calculation of metabolites per Gm. of creatinine.

† The dose of T_3 was 200 μg. daily for a period of from ten to twenty-one days prior to the study, except for Subject SR who received the hormone for five days prior to, as well as during, a four-day collection.

thyronine (T_3). Four of these control subjects were schizophrenic patients. Table 3 shows a comparison of the results obtained in the 4 mental patients, the 6 normal subjects, the total control series, and in all subjects (mean value) studied by Romanoff and collaborators (6). The close agreement between the values for the normal subjects and those for the schizophrenic patients is evidence that the mental disturbance of these patients

TABLE 2. ENDOGENOUS HYDROCORTISONE METABOLITES IN EUTHYROID SUBJECTS UNDER CONTROL CONDITIONS AND AFTER TREATMENT WITH TRIIODOTHYRONINE

Subject and treatment	Metabolites (mg./24 hrs./Gm. creatinine)			
	THE	THF	ATHF	Total
BS(28F)	1.6	1.6	0.6	3.8
T_3 therapy	1.8	1.4	1.0	4.2
BE(33M)	2.5	1.3	0.7	4.5
T_3 therapy	4.8	1.5	1.4	7.7
SR(23M)	1.1	1.2	0.9	3.2
T_3 therapy	2.2	1.3	1.4	4.9
WN(23F)	1.9	1.2	0.5	3.7
T_3 therapy	3.3	1.1	1.1	5.5
VN(24M)	1.9	1.1	0.3	3.3
T_3 therapy	1.5	0.8	0.5	2.8
SZ(24M)	1.3	1.3	0.7	3.3
T_3 therapy	2.1	1.0	1.0	4.1
ML(27F)	1.9	1.7	1.2	4.8
FR(32M)	1.6	1.1	0.5	3.2
GR(41M)	1.2	1.4	0.7	3.3
MN(58M)	2.1	2.1	1.0	5.2

did not interfere with their production or metabolism of hydrocortisone. This conclusion is in complete agreement with the earlier report of Romanoff *et al.* (7). It is striking that there was such close correspondence between the data on the 40 subjects of Romanoff and collaborators and the data of the present series, especially in view of the fact that different methods of enzymatic hydrolysis, different separation schemes, and different colorimetric evaluation procedures were employed in the 2 studies. These results, therefore, justify the use of schizophrenic patients in the present investigation.

It can be seen from Tables 2 and 4 that increased production of thyroid hormone, whether resultant from the administration of triiodothyronine or from spontaneous hyperthyroidism, caused an increase in the total produc-

TABLE 3. COMPARISON OF ENDOGENOUS HYDROCORTISONE METABOLITES IN VARIOUS CONTROL SUBJECTS

	Creatinine excretion (Gm./24 hrs.)	Metabolites (mg./24 hrs./Gm. creatinine)			
		THE	THF	ATHF	Total
All subjects	1.41	1.7	1.4	0.7	3.8
Mental patients	1.35	1.8	1.3	0.7	3.8
Normal subjects	1.46	1.7	1.5	0.7	3.9
All subjects, Romanoff *et al.* (6)	1.25	1.8	1.1	0.7	3.6

tion of hydrocortisone as measured by the 3 hydrocortisone metabolites. The 6 subjects who received triiodothyronine showed an increase in production of hydrocortisone of more than 30 per cent as judged by the levels of these 3 metabolites. The "normal" response of the small schizophrenic group to the administration of thyroid hormone, as measured by hydrocortisone production and metabolism, indicates that schizophrenics are sensitive to this type of stimulus. It has been suggested (8) that the reduced affective behavior of the schizophrenic is also accompanied by reduced physiologic, biochemical and pharmacologic changes. Our results with thyroid hormone administration fail to support this hypothesis. The increase in hydrocortisone production was still more striking in the patients with spontaneous hyperthyroidism; the total excretion of the 3 metabolites

TABLE 4. ENDOGENOUS HYDROCORTISONE METABOLITES IN SPONTANEOUS HYPERTHYROIDISM BEFORE AND AFTER TREATMENT

Subject and treatment	Metabolites (mg./24 hrs./Gm. creatinine)			
	THE	THF	ATHF	Total
KS(68M)	6.4	2.8	1.5	10.7
Euthyroid	0.8	1.3	0.2	2.3
LB(61F)	5.3	2.6	1.6	9.5
ML(44F)	7.6	2.0	1.8	11.4
Euthyroid	1.3	0.8	0.3	2.4
DO(43M)	5.2	1.4	0.8	7.4
ACTH	12.7	7.1	5.7	25.5

was approximately double that in the control series. It should be emphasized that these results were obtained on patients who were previously untreated for their hyperthyroidism. In 2 patients (not reported in this paper) who had been treated with propylthiouracil for prolonged periods and then allowed to become clinically hyperthyroid, the alteration in metabolism of endogenous hydrocortisone was evident (*i.e.*, THE greater than THF) but the total amount of the 3 metabolites was within the normal range. This problem will be studied further. It is noteworthy, however, that cats and rats treated with thiouracil exhibited some adrenocortical atrophy (9, 10).

A high level of circulating thyroid hormone was characterized by a considerable absolute and relative increase in the formation of THE. There was a lesser increase in ATHF, again more marked in the patients with spontaneous hyperthyroidism than in those treated with triiodothyronine. The absolute amount of THF was unchanged in the triiodothyronine-treated group, but this represented a smaller fraction of the total metabolites examined, as evident in Table 2. In the patients with spontaneous

hyperthyroidism, THF excretion was at a slightly higher level than in the control series but again represented a smaller fraction of the total. The percentage of the total steroids represented by THF in spontaneous hyperthyroidism was precisely comparable to that in the patients treated with triiodothyronine. It should be noted that the results were the same whether the whole series of control subjects was compared with the treated patients, or individual subjects were compared before and after administration of triiodothyronine.

Table 4 includes data on the response of a hyperthyroid patient (DO-43M) to ACTH. ACTH (20 units) was administered by continuous intravenous infusion for eight hours daily for three consecutive days. The results shown are the average daily endogenous production for three days preceding this treatment and for the third day of tropic hormone administration. It is evident that the greatest increase in hydrocortisone metabolites was manifest in THE. This contrasts strikingly with the response of normal subjects or patients with various disorders to similar treatment with ACTH. In more than 50 such studies made in these laboratories[2] this hyperthyroid patient (DO-43M) was the only subject in whom the increase of THE production was found to exceed that of THF production. In all other patients, THF was predominant over THE by from 25 to 500 per cent.

In myxedema, as shown in Table 5, the total production of hydrocortisone was materially diminished when compared with that in the euthyroid subjects. Together with this decrease there was an alteration in the route of metabolism in the opposite direction to that found in spontaneous or induced hyperthyroidism. In the myxedema patients, THF was the principal metabolite at more than double the level of THE. The amount of ATHF was about half that found in the control patients but constituted almost precisely the same percentage of the total of the 3 metabolites. The results are summarized in Table 6.

The response of a hypothyroid patient (Table 5, WY-65F) was studied with ACTH in the manner described for the subject with spontaneous hyperthyroidism. Despite the relatively low basal level of hydrocortisone production, there was a sharp increase by the third day of treatment and, as anticipated, the chief metabolite was THF, which exceeded THE by approximately 200 per cent. The contrast in the peripheral metabolism of hydrocortisone under maximal adrenal stimulation between these two extremes of thyroid function is impressive.

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[2] Gallagher, T. F., and Spencer, H.: unpublished results. For comparable data see, among others, Gold *et al.* (11), and Gallagher (12).

TABLE 5. ENDOGENOUS HYDROCORTISONE METABOLITES IN MYXEDEMA BEFORE AND AFTER TREATMENT

Subject and treatment	Metabolites (mg./24 hrs./Gm. creatinine)			
	THE	THF	ATHF	Total
MA(42F)	0.5	1.3	0.3	2.1
Euthyroid	3.5	1.5	0.9	5.9
SA(60M)	0.7	1.4	0.4	2.5
WE(59F)	0.5	0.9	0.4	1.8
WR(68M)	0.5	1.1	0.2	1.8
CR(72F)	0.5	0.8	0.2	1.5
GZ(60F)	0.4	1.5	0.6	2.5
KA(34F)	0.5	0.9		1.4
WY(65F)	0.6	0.9	0.1	1.6
ACTH	4.7	14.5	1.1	20.3

Tracer studies of metabolism of exogenous hydrocortisone

Table 7 shows the extent of transformation of a tracer dose of hydrocortisone to THE, THF and ATHF in 4 of the control subjects in the euthyroid state and when treated with triiodothyronine. Each subject exhibited an increased transformation of the hormone to THE together with a diminution in the amount metabolized to THF. The differences observed in the amount of ATHF produced are of questionable significance. It is pertinent that the total amount of these metabolites formed from the tracer dose was not significantly different in euthyroidism or during triiodothyronine administration; therefore, the changes observed in THE and THF represent alterations in the metabolic route without diversion to other metabolites.

Table 8 shows the fate of a tracer dose of hydrocortisone in 4 patients with spontaneous hyperthyroidism, and similar studies in 3 of these pa-

TABLE 6. ENDOGENOUS HYDROCORTISONE METABOLITES AT DIFFERENT LEVELS OF THYROID HORMONE ACTIVITY

Subjects	Metabolites						
	mg./24 hrs./Gm. creatinine				per cent of toal		
	THE	THF	ATHF	Total	THE	THF	ATHF
Untreated	1.8	1.3	0.6	3.7	48.6	35.1	16.2
T_3-treated	2.7	1.1	1.1	4.9	55.1	22.4	22.4
Myxedema	0.5	1.1	0.3	1.9	26.3	57.9	15.8
Hyperthyroidism	6.1	2.2	1.4	9.7	62.9	22.7	14.4

TABLE 7. METABOLITES OF EXOGENOUS HYDROCORTISONE-4-C^{14} IN EUTHYROID SUBJECTS BEFORE AND DURING TREATMENT WITH TRIIODOTHYRONINE

Subject and treatment	Metabolites (per cent)*			
	THE	THF	ATHF	Total
BS(28F) control	19	15	8	42
T_3 therapy	32	10	7	49
BE(33M) control	32	18	8	58
T_3 therapy	41	12	4	57
SR(23M) control	20	14	13	47
T_3 therapy	27	8	12	47
WN(23F) control	29	15	8	52
T_3 therapy	41	9	6	56

* Per cent refers to the neutral steroid fraction after enzymic hydrolysis. The subjects were given approximately 1 μC of hydrocortisone-4-C^{14}. The recovery of radioactivity within twenty-four hours averaged 79% and ranged from 72% (Subject BS during T_3 therapy) to 87% (Subject SR, during T_3 therapy). The hydrolysis averaged 71% and ranged from 51% (Subject SR, control period) to 75% (Subject BS, control period).

tients after they had been treated. It is evident that spontaneous hyperthyroidism is associated with the same alteration in metabolism that was demonstrated with administration of triiodothyronine. Moreover, as was true for the endogenous production, the metabolic alteration was more marked in the patients with spontaneous hyperthyroidism. Treatment of these patients so that they became either euthyroid or slightly myxedematous altered their steroid metabolism in the expected direction, in that the fraction of THE was diminished and THF was increased—again without significant alteration in ATHF.

The results of tracer studies in patients with myxedema are shown in

TABLE 8. METABOLITES OF EXOGENOUS HYDROCORTISONE-4-C^{14} IN SPONTANEOUS HYPERTHYROIDISM BEFORE AND AFTER TREATMENT

Subject and treatment	Metabolites (per cent)*			
	THE	THF	ATHF	Total
KS(68M)	27	15	5	47
Euthyroid	18	24	5	47
ML(44F)	45	8	4	57
Euthyroid	26	14	7	47
GR(67M)	26	7	4	37
Euthyroid	26	18	6	50
PK(46F)	39	9	6	54

* Per cent refers to the neutral steroid fraction after enzymic hydrolysis. The subjects were given approximately 1μC of hydrocortisone-4-C^{14}. The recovery of radioactivity within twenty-four hours averaged 78% and ranged from 61% (Subject KS, euthyroid) to 92% (Subject PK). The hydrolysis averaged 63% and ranged from 54% (Subject GR, untreated) to 68% (Subject KS, both periods).

TABLE 9. METABOLITES OF EXOGENOUS HYDROCORTISONE-4-C^{14} IN MYXEDEMA

Subject and treatment	Metabolites (per cent)*			
	THE	THF	ATHF	Total
MA(42F)	17	29	2	48
Treated	38	14	11	63
SA(60M)	16	40		56
WE(59F)	17	28	2	47
WR(68M)	18	35	3	56
CR(72F)	17	35	3	55
RS(65M)	20	29	2	51
Second study†	15	35	3	53
LN(77F)	16	36	4	56

* Per cent refers to the neutral steroid fraction after enzymic hydrolysis. The subjects were given approximately 1 μC of hydrocortisone-4-C^{14}. The recovery of radioactivity averaged 62% and ranged from 50% (Subject RS, second study) to 77% (Subject MA). The hydrolysis averaged 54% and ranged from 32% (Subject SA) to 67% (Subject MA, untreated).

† Second study two months after the first; at that time the patient was clinically as myxedematous as before. During the second study he was receiving 50 mg. of testosterone per day intramuscularly.

Table 9. Hypothyroidism was associated with an increase in the relative conversion of hydrocortisone to THF and a diminished production of THE. In this series of patients, as well as in the patients with spontaneous hyperthyroidism, it is important to note that the total percentage of the hormone transformed to the 3 metabolites studied was essentially the same as in the euthyroid subjects.

Table 10 summarizes these observations on the metabolic transformation

TABLE 10. COMPARISON OF METABOLITES OF EXOGENOUS HYDROCORTISONE-4-C^{14} AT VARIOUS LEVELS OF THYROID HORMONE ACTIVITY

Subjects	Metabolites						
	Per cent*				Per cent of total		
	THE	THF	ATHF	Total	THE	THF	ATHF
Controls	25	16	9	50	50	32	18
Normal men, published data (5)	24	18	10	52	46	35	19
T_3-treated	35	10	8	53	66	19	15
Hyperthyroid	34	10	5	49	69	20	10
Myxedema	17	33	3	53	32	62	6

* Per cent refers to the neutral steroid fraction after enzymic hydrolysis.

of exogenous hydrocortisone and also includes a comparison with a series of normal men similarly studied (5). The agreement between the present findings in schizophrenic control patients and the results published for 5 normal men is impressive. This reinforces the other evidence that the metabolism of hydrocortisone in these patients is indistinguishable from that in the normal subject. It is evident from Table 10 that induced or spontaneous hyperthyroidism was associated with a shift of metabolism away from THF and in the direction of THE without too great alteration

TABLE 11. COMPARISON OF CORTOLS, CORTOLONES AND 17-KETOSTEROIDS FROM EXOGENOUS HYDROCORTISONE-4-C^{14} AT DIFFERENT LEVELS OF THYROID HORMONE ACTIVITY

Subject	Metabolites (per cent)*			
	Cortols	Cortolones	OH-E	11-OE
PS —Untreated	9.1	23.0	1.7	2.0
T_3	3.7	25.0	2.1	4.0
BE —Untreated	4.0	21.0	1.0	0.2
T_3	2.0	23.0	2.2	4.8
SR —Untreated	4.8	15.8	2.5	2.8
T_3	2.9	20.0	2.6	9.3
WN—Untreated	4.9	21.0	2.1	3.4
T_3	2.7	23.0	3.5	11.0
GR —Treated	6.0	24.0	1.0	0
Hyperthyroid	4.6	30.0	0.7	0.5
ML—Treated	4.2	22.0	5.9	7.5
Hyperthyroid	1.5	21.0	1.0	5.6
KS —Treated	6.7	20.5	0.5	0.5
Hyperthyroid	3.5	25.0	1.0	0
MA—Myxedema	12.0	16.0	11.0	7.5
Euthyroid	4.1	20.0	1.0	2.3

* Per cent refers to the neutral steroid fraction after enzymic hydrolysis.

in transformation to ATHF; in myxedema, however, the amount of THE was significantly diminished.

The more completely reduced metabolites of hydrocortisone, the cortols and the cortolones, were examined in 8 of these patients at two different levels of thyroid hormone. Since no attempt was made to separate the individual isomers of these two classes, the values in Table 11 represent the sum of the epimers at C-20. It is evident that in each subject examined, an increase in thyroid hormone was accompanied by a decrease in the conversion of hydrocortisone to the cortols. Conversely, the cortolones increased in each individual subject except ML, in whom conversion was unchanged when the thyroid hormone level was elevated. The changes in these metabolites with respect to the oxidized or reduced form at C-11 are analogous to

those observed with the tetrahydro compounds and, indeed, may be a reflection of similar changes observed with the reducing ketols. It should be noted that the cortols and cortolones comprised from 20 to 35 per cent of the total metabolites in the neutral steroid fraction in the tracer studies, and that when added to the values for THE, THF and ATHF, from 70 to 85 per cent of the metabolites of hydrocortisone were identified and measured.

The 2 principal 17-ketosteroid metabolites of hydrocortisone are 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone. In general, as can be seen from Table 11, these products exhibited the same alteration in metabolism observed with the C-21 metabolite. As measured by the fate of a tracer dose, there were marked changes in 11-ketoetiocholanolone when the production of this metabolite was examined in the same subject while euthyroid and while treated with triiodothyronine. The increase in thyroid hormone resulting from administration of triiodothyronine produced an elevation in both the endogenous production and the percentage conversion of a tracer dose. With 11-hydroxyetiocholanolone the results were inconclusive.

The patients with spontaneous hyperthyroidism failed to exhibit any significant change in the conversion of exogenous hydrocortisone to 11-ketoetiocholanolone after they were made euthyroid. Two of these patients, however, exhibited the expected rise in 11-hydroxyetiocholanolone after effective treatment of their dysthyroidism. Treatment of the patient with myxedema resulted in a fall in the percentage conversion to 11-hydroxyetiocholanolone.

DISCUSSION

These data concerning changes in hydrocortisone metabolism in response to alterations in the level of thyroid hormone, when considered together with the earlier studies of androgen metabolism (13), indicate that 2 quite different biochemical transformations of steroid molecules are consistently affected by the thyroid. The effect observed with C_{19} compounds characteristic of androgenic steroids involves alteration in the stereochemical course of reduction of C-5 so that, with excess of thyroid hormone, there is increased production of 5α metabolites. As there is also increased formation of the 5α reduction products of hydrocortisone in response to thyroid hormone, this appears to be a consequence of an absolute increase in hydrocortisone production rather than of altered peripheral metabolism, since studies with C^{14}-hydrocortisone reveal no change in the proportion of 5α metabolites found in hyperthyroidism. The new biochemical effect described in this study, characteristic of the influence of thyroid hormone on the metabolism of hydrocortisone, involves changes in the state of oxidation or reduction at C-11.

The significance of these alterations in the metabolism of hydrocortisone can be considered in terms of the reactions shown diagrammatically in Figure 1. Particular attention is drawn to the fact that at successive stages of the *in vivo* transformation of hydrocortisone, the oxygen function at C-11 may be reversibly reduced to a hydroxyl group or oxidized to a ketone. From these studies, then, an excess of thyroid hormone enhances the cellu-

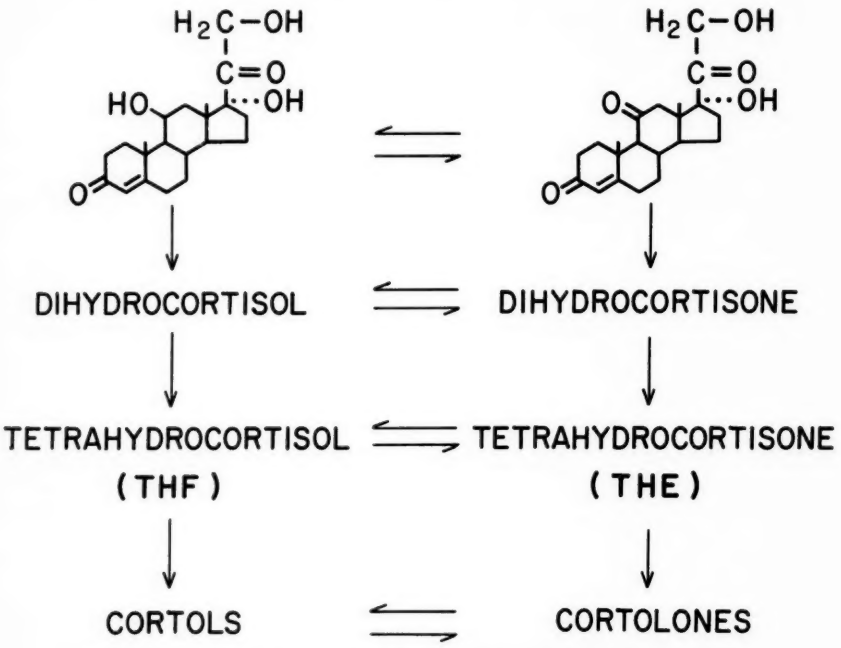

FIG. 1. Potential pathways of hydrocortisone metabolism in man.

lar processes which lead to the production of the oxidized form at C-11; that is, the principal metabolites are shifted to the right side of Figure 1, whereas a deficiency leads to more of the reduced compounds shown on the left side of Figure 1. This change in the character of the metabolites unquestionably results from alteration in peripheral metabolism, since this was manifest with tracer doses of exogenous hydrocortisone as well as with endogenous hormone production.

Striking evidence of a change in the peripheral metabolism of hydrocortisone induced by thyroid hormone is seen in the study in which adrenocorticotropin was administered to a subject with hyperthyroidism. The increased quantity of endogenously secreted hydrocortisone was metabolized in the pattern that must now be regarded as typical of the effect of

thyroid hormone on corticoid metabolism, *i.e.*, THE was the major metabolite. This is in marked contrast to the usual pattern of hydrocortisone metabolism in Cushing's syndrome and following administration of ACTH to euthyroid subjects. When hydrocortisone production is increased in these latter circumstances, THF is always the major urinary metabolite and the relative production of THE declines.

The significance of the observed alteration in hydrocortisone metabolism lies in the probable biologic inactivity of cortisone and the 11-keto metabolites altered in ring A. This inactivity had been suspected on the basis of the poor clinical response to cortisone applied topically in areas with relatively deficient circulation *e.g.*, intra-articularly (14). This conclusion has been confirmed by careful studies of the biologic effects of 2-methylated corticosteroids. Liddle, Richard and Tomkins (15), and Dulin, Bowman and Stafford (16) demonstrated that the 2-methyl analogue of hydrocortisone was very much *more* potent, biologically, than the parent compound, whereas the 2-methyl analogue of cortisone was very much *less* potent than cortisone. Bush and Mahesh (17) showed in man that there was little interconversion of the 11β-hydroxyl and the 11-keto group of the 2α-methyl corticoids and stated ". . . it seems clear that this exceptionally low activity of the 11-ketone is not easily accounted for except by supposing that it is itself inactive at the receptor site." These findings are consistent with the conclusion that 11-ketocorticosteroids become active only by conversion to the 11-hydroxy compound or, in other words, the 11-keto compounds are intrinsically inactive. What little biologic activity might reside in cortisone itself would be further weakened by its shorter biologic half-life compared to that of hydrocortisone (18).

The alterations in adrenal secretory rate also observed are clearly at the glandular level and, granting the biologic inactivity of 11-keto derivatives of hydrocortisone, would follow from the operation of a negative feedback control of pituitary ACTH secretion. The diversion of hydrocortisone to 11-keto derivatives incapable of suppressing the pituitary would increase secretion of ACTH. The mechanism would operate until the absolute amount of hydrocortisone, and perhaps its related 11-hydroxy metabolites, was sufficient to bring the pituitary adrenal system into a new level of homeostasis. Thus the increased production of hydrocortisone found in hyperthyroidism would be a consequence of the efficient oxidation of the 11β-hydroxyl group, evident from the increment in 11-ketosteroids. In hypothyroidism, on the other hand, the oxidation of the 11-hydroxyl group is sluggish, so that more hydrocortisone and its 11-hydroxylated relatives are available for pituitary suppression. Therefore, adrenocortical turnover would be maintained at a lower level than in the euthyroid subject. In both dysthyroid states the operation of the postulated feedback mechanism

would result in a normal level of plasma hydrocortisone, so that the patients in this sense would remain eucorticoid despite the altered rate of hydrocortisone production. In this connection it is of interest that Williams, Crispell and Parson reported a diminished rate of metabolism of corticoids in primary myxedema (19).

Our data show a definitely deranged production of hydrocortisone in dysthyroid states and are in agreement with earlier reports (20–22). The fact that Peterson (21) found the plasma hydrocortisone level to be normal in hypo- and hyperthyroidism suggested that the altered production of hydrocortisone was a secondary change compensatory for a primary disturbance in its peripheral metabolism. Peterson left open the question of the nature of that disturbance; the present study indicates that the oxidative mechanism converting hydrocortisone to inactive 11-keto metabolites is the step which initiates the entire observed sequence of events.

The chemical and clinical signs of adrenal insufficiency associated with thyroid "storm" may well represent a breakdown of the homeostatic mechanism. The heightened level of thyroid function which results in a "storm" may be viewed as inducing a state of adrenal insufficiency, even in the face of increased hydrocortisone production, by stimulating metabolic inactivation of hydrocortisone by the formation of steroid metabolites (*i.e.*, 11-keto compounds) which lack the complete biologic activity characteristic of the hormone. Treatment of the "storm" with adrenocortical hormones would then represent replacement therapy, and has in fact been found to be quite effective (23).

These differences in the pattern of metabolism of the increased quantities of hydrocortisone produced in hyperthyroidism as compared to the pattern resultant from spontaneous or induced Cushing's syndrome probably account for the failure of typical Cushing's syndrome to develop in hyperthyroid patients in spite of suitably elevated levels of hydrocortisone production. The increased transformation of hydrocortisone to its 11-ketonic metabolites in hyperthyroidism may account for the lack of development of the facies and habitus characteristic of increased hydrocortisone production in Cushing's syndrome. This consideration would imply that certain biologic effects such as the production of buffalo hump, moon facies and the like were direct properties of *hydrocortisone* and not shared with its metabolites. These physical signs do not appear in hyperthyroidism because the elevated hydrocortisone secretion is dissipated by metabolic transformation, and the "effective" quantity of hydrocortisone is maintained at the normal level.

As far as certain other features of corticoid excess in hyperthyroidism are concerned, the situation is more equivocal. Similar clinical and biochemical changes occur both in Cushing's syndrome and in hyperthyroidism, *e.g.*,

metabolic bone disease (osteoporosis), negative nitrogen balance, muscle wasting, creatinuria, and hyperglycemia; moreover, some patients with Cushing's syndrome have exophthalmos. Since hydrocortisone production is increased in hyperthyroidism, it is tempting to speculate that some, but not necessarily all, the features of hyperthyroidism which are similar to those found in Cushing's syndrome may be the direct consequence of the excess of hydrocortisone and its metabolites (including cortisone) and only indirect or distal effects of thyroid hormone.

It is of some interest to compare these *in vivo* findings in man with the *in vitro* studies of McGuire and Tomkins (24), which dealt with the effect of thyroid hormone on the metabolism of steroids by rat liver homogenates. These authors, in their system, found that the effect of thyroid hormone on the metabolism of testosterone was in agreement with the previous *in vivo* findings in man. On the other hand, their results with hydrocortisone indicated a predominant effect of thyroid hormone on 5α reduction, which is not seen in man. Our finding of increased oxidation of the 11-hydroxyl group to an 11-ketone was not observed in their system. This is almost certainly one of the unpredictable species differences so often found in the study of steroid hormone metabolism. It is perhaps accentuated in this case by the fact that hydrocortisone is essentially a "foreign" hormone to the rat since it is only a minor constituent of its adrenal secretion, corticosterone being the major secretory product.

These metabolic effects on steroid hormones are perhaps the most distinctive and reproducible of any of the biochemical consequences of thyroid hormone activity. They can be readily and reproducibly measured, and provide a pattern of steroid hormone metabolites, characteristic of the thyroid status. In any event, it is clear that major targets of thyroid hormone action are the enzyme systems that metabolize the hormones of the adrenal cortex, both the "androgen or dispensable" component and hydrocortisone, the "indispensable" component.

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THE EFFECT OF EUTHYROIDAL HYPERMETABOLISM ON CORTISOL REMOVAL RATES

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ABSTRACT

Cortisol secretion and removal rates were determined in 6 leukemia patients during a hypermetabolic phase of their disease, and again when the metabolic rate was normal following appropriate chemotherapy. The hypermetabolic phase was associated with an increased rate of secretion of cortisol and a normal or diminished plasma removal rate. This suggests that the accelerated plasma cortisol disappearance rates in hyperthyroidism are probably not due to hypermetabolism *per se*, but due to specific effects of the thyroid hormone.

IN THYROTOXICOSIS, the rate of disappearance of cortisol from plasma is accelerated (1–3) and the secretion of cortisol is increased (4). *in vitro* studies of rat tissues have demonstrated that thyroid hormone accelerates the rate of reduction of the C_4-olefin group of cortisol by two mechanisms: a) increasing the available reduced triphosphopyridine nucleotide, and b) increasing hepatic microsomal cortisol-5α-hydrogenase (5, 6).

On the basis of their clinical studies, Brown *et al.* (2) suggested that the rate of cortisol removal was directly proportional to the basal metabolic rate. In no instance however, was the specificity of the thyroid effect established. For this reason we have examined several aspects of cortisol metabolism in a group of patients with euthyroidal hypermetabolism, and subsequently when the metabolic rate was normal.

Plan of study. The cortisol secretion rate and its removal rate from plasma were determined in patients with leukemia during hypermetabolic and eumetabolic phases of their disease, the latter phase resulting from appropriate chemotherapy. These states were defined by repeated determinations of the basal metabolic rate (BMR). Six sets of satisfactory studies were obtained on subjects who fulfilled the requirements, which were: 1) a BMR higher than +30 per cent, and at some other time a normal BMR; 2) normal findings with liver function tests; 3) absence of infection 4) normal renal function; and 5) normal thyroid function.

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METHODS

Each patient was hospitalized at least twenty-four hours prior to the determination of the BMR with a Sanborn Metabulator. The serum level of protein-bound iodine was measured before each study. The following liver function tests were performed before each study; 45-minute bromsulfalein retention, direct and indirect determination of serum bilirubin, thymol turbidity and serum levels of albumin and globulin. The concentration of blood urea nitrogen was normal in each case. The status of each subject is presented in Table 1.

The plasma cortisol removal rate was determined using cortisol-4-C^{14} with a specific activity of 21.7 μc. per mg., obtained from the Endocrinology Study Section of the National Institutes of Health. A 2-μc. sample dissolved in ethanol was diluted with 30 ml. of physiologic sodium chloride and injected intravenously over a period of three to five minutes. Blood samples were obtained at 30, 60, 90, 150 and 240 minutes after injection, centrifuged immediately, and the plasma stored at 5° C. until the extraction procedure was started. The plasma was processed in order to extract free cortisol and its metabolites according to the method of Peterson *et al.* (7). For each patient the disappearance rates with their standard errors were estimated by fitting straight lines relating the logarithm

TABLE 1

Patient age (yrs.) & sex	Diagnosis*	Study No.	Serum protein-bound iodine (μg./100 ml.)	Medication†
J.H.(6M)	ALL	1	8.6	Methotrexate.
		2	8.6	Methotrexate.
D.W.(8F)	ALL	1	5.2	Untreated.
		2	5.9	Methotrexate.
B.C.(9M)	ALL	1	6.4	Methotrexate+6-mercaptopurine.
		2	6.0	Cytoxan.
O.M.(43F)	CML	1	5.0	Untreated.
		2	5.9	Trimethylcolchicinic acid.
J.G.(12M)	ALL	1	7.2	Untreated.
		2	7.2	6-Mercaptopurine.
J.W.(19M)	ALL	1	4.7	Untreated.
		2	4.9	Methotrexate.
A.K.(41M)	T	1		10-day withdrawal of T_3.
		2		125 μg. T_3 daily.
D.P.(18F)	T	1		14-day withdrawal of T_3.
		2		125 μg. T_3 daily.
W.P.(36F)	T	1		14-day withdrawal of T_3.
		2		125 μg. T_3 daily.
T.M.(44F)	H	1		30 mg. cortisol daily.
Y.A.(52F)	H	1		30 mg. cortisol daily.

* ALL = Acute lymphocytic leukemia.
CML = Chronic myelogenous leukemia.
T = Thyroidectomized thyroid carcinoma.
H = Hypophysectomized for carcinoma of the breast.
† T_3 = Triiodothyronine.

of the counts per minute per ml. of plasma to the time after administration, using the method of least squares. The change in the half-time was tested using Student's t test, and significance was assumed when $P < 0.05$ (8).

A 24-hour urine was collected following injection of the labeled cortisol. The cortisol secretion rate was estimated by the method of Cope and Black (9), and Silber-Porter chromogens (17,21-dihydroxy-20-ketosteroids) by a modification of the S-P method (10). Secretion rates were calculated from the specific activities of urinary tetrahydrocortisone and tetrahydrocortisol after β-glucuronidase hydrolysis, extraction, and chromatography on the Bush B5 system, generally preceded by chromatography in the toluene-propylene glycol system. Total recovery of radioactivity was not estimated in the urine so that the results may be uncertain by up to 20 per cent (7).

All analyses for carbon^{14} were carried out with the Packard liquid scintillation spectrometer, using a toluene DPO (diphenyloxazole) phosphor. With a background of 25 cpm, the efficiency for carbon^{14} was approximately 60 per cent.

To determine the validity of this method for estimating the secretion rate of cortisol, the rate was determined in 2 hypophysectomized women receiving 30 mg. of cortisol daily in multiple fractional doses. The estimated secretion agreed satisfactorily with the daily dose of cortisol (Table 1, Patients T.M. and Y.A.).

To establish that these methods would demonstrate the effect of thyroid hormone, the plasma cortisol disappearance rate was determined in 3 thyroidectomized patients: initially when replacement therapy was withdrawn, and again following a period of triiodothyronine therapy (Tables 1 and 2, Patients A.K., D.P. and W.P.). The rate of removal of cortisol was significantly greater following treatment with triiodothyronine (Table 2, Patients A.K., D.P. and W.P.; Fig. 1)

RESULTS

The rate of disappearance of cortisol was not increased in any of the hypermetabolic patients with leukemia despite elevation of the BMR to +62 per cent (Table 2). In fact, the biologic half-time of carbon^{14} was slower in 5 of the 6 patients during the hypermetabolic phase. In 3 of these 5 subjects, the decrease in half-time was significant ($P < 0.05$) (Fig. 1).

The excretion of Silber-Porter chromogens was higher during the hypermetabolic phase. Likewise cortisol secretion rates were increased during the hypermetabolic phase relative to those during the eumetabolic phase. Two of the 6 patients (J. G. and J. W.) were febrile during their initial study. The BMR repeated within two days was still elevated, although the patients had become afebrile without treatment. The results in these patients did not differ from the others.

DISCUSSION

The hypermetabolic state was accompanied by an increased secretion of cortisol, as estimated either by excretion of Silber-Porter chromogens or by the more direct method of Cope and Black, whereas the plasma cortisol removal rate was either unchanged or slowed.

The tendency toward normal or reduced disappearance rates of cortisol during the hypermetabolic state recalls studies of cortisol metabolism in a

variety of nonspecific stress situations. Dogs subjected to "stress" demonstrated a normal or slower removal rate (11). Urquhart *et al.* (12) showed that the liver of the "stressed" rat had a diminished capacity to metabolize steroids *in vitro*. Surgical stress (13) and septicemia (14) were associated with either a normal or diminished plasma cortisol disappearance rate in man. In the latter study, the relative roles of the rate of cortisol secretion

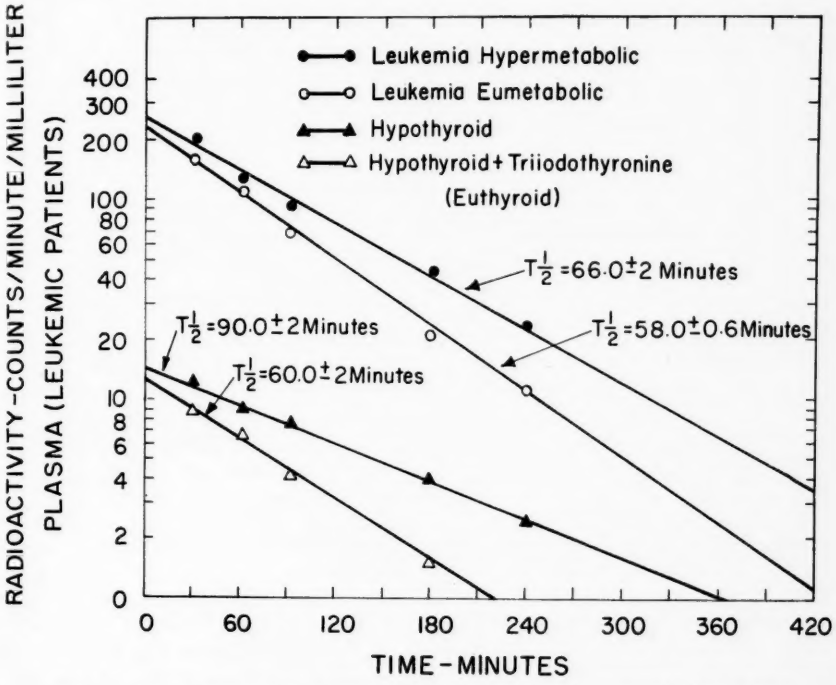

FIG. 1. Rate of removal of cortisol from plasma in various metabolic states.

and cortisol removal in producing the elevated plasma cortisol levels were not assessed.

These data suggest that the pattern of cortisol metabolism during the hypermetabolic phase of leukemia falls into that rather amorphous category of "stress reaction" characterized by increased adrenocortical secretion and decreased or normal cortisol removal. In view of the demonstrated acceleration of plasma cortisol removal rates in hyperthyroidism, it seems probable that this effect is not secondary to the increased metabolic rate but is attributable specifically to an action of thyroid hormone.

The possibility that leukemic infiltration of the liver accounted for the decreased cortisol removal rates is rendered unlikely by the normal clear-

TABLE 2

Patient	Study No.	BMR (%)	C^{14} half-time (mins.)	P*	Urinary Silber-Porter chromogens (mg./24 hrs.)	Cortisol secretion (mg./24 hrs.)	
						Tetra-hydro E	Tetra-hydro F
J.H.	1	+ 3.0	73.0 ±2†		2.1	6.6	5.7
	2	+39	100.0 ±5	< .05	3.3	10.4	8.8
J.W.	1	+35	66.0 ±2		4.2	10.0	—
	2	+15	58.0 ± .6	< .05	2.1	4.3	—
B.C.	1	+35	67 ±3		—	16.0	—
	2	+ 6	76 ±3	> .05	—	11.0	—
O.M.	1	+45	83 ±6		8.4	26.0	30.0
	2	+ 9.0	77 ±3	> .05	5.3	17.0	15.0
J.G.	1	+49	68 ±4		3.3	10.4	8.8
	2	+ 3.0	49.0 ±1	< .05	2.1	6.6	5.7
J.W.	1	+62	133 ±13		10.1	22	20
	2	− 1.0	120 ±8	> .05	7.4	17	14
A.K.	1	−13	90.0 ±2		—	—	—
	2	+ 5.0	60.0 ±2	< .01	—	—	—
D.P.	1	−19	86.0 ±4		6.2	—	—
	2	+ 3	46.0 ±2	< .01	11.5	—	—
W.P.	1	−29	93.0 ±5		4.1	18	17
	2	+ 5	53 ±2	< .01	8.9	27	31
T.M.	1	—	—	—	13.5	26	24
Y.A.	1	—	—	—	15.8	27	26

* Probability that the differences in the half-times were due to chance.
† Standard error of the estimate.

ance of bromsulfalein and the other normal findings with liver function tests. The effect of chemotherapeutic agents on cortisol metabolism is unknown. However, the variety of drugs used and the fact that 2 patients (J. H. and B. C.) received chemotherapy during both studies, make it improbable that chemotherapy greatly influenced the metabolism of cortisol.

Acknowledgment

It is a pleasure to acknowledge our gratitude to Dr. J. D. Davidson for his many helpful suggestions. We wish to thank Mrs. Barbara Riter for her skillful technical assistance, and Miss Joan Gurian of the Biometry Branch, National Cancer Institute, for guidance with the statistical analysis.

Addendum

Through the courtesy of Prof. Rolf Luft, Karolinska Hospital, Stockholm, we were able to investigate the cortisol removal rate in a unique patient with a BMR of +170 to +200 per cent, normal thyroid function, and normal renal and hepatic function. The carbon^{14} half-time was 84 minutes—a result well within the normal range.

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ADVENTURES IN CLINICAL RESEARCH ON BONES AND STONES

Presidential Address—The Endocrine Society*

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IN RISING to say a few words to you as retiring President of The Endocrine Society, it cannot be said that there has not been ample warning of this moment. For two years I have known that only an act of God could avert the necessity of my speaking at this time and of your being a captive audience.

All sorts of notions have passed through my mind for subject matter with which to harangue you. One might philosophize on how to chart the course of this Society of ours, with its wide mixture of disciplines. In this connection the obvious could be cited, that our goal is to bring medicine ever closer to being an exact science while still preserving its art and thus maintaining the friendly and revered position which physicians have enjoyed for so long in our society. One fears sadly that as scientific medicine has flowered, much of our former prestige has already been lost, and we are now looked upon as following a trade rather than a profession. One might also give cogent reasons for wishing to maintain the unity of our Society, urging that there be one journal rather than two, so that the clinician would be almost compelled to read of laboratory doings and the laboratory might continue to receive the full stimulus of the clues to further understanding that nature provides so prolifically in sick persons.

Another possible subject for discourse was a recollection of personalities in the world of endocrine investigation whom I have met along the way. Or an attempt might have been made at a learned discussion of the moral obligations of the medical scientist, to society in general and to the human sick in particular. None of these seemed to strike the right chord; and so I turned to reflect on some of the bizarre, and at times downright silly, wisps of intellectual synthesis which have motivated certain phases of the investigative efforts of our own little group. This tale might be entitled "On Bones, Stones and Concrete"—the last named substance being descriptive perhaps of the author's brain.

In 1927 Fuller Albright was induced by Read Ellsworth to spend a year at Johns Hopkins. Through the fortunes of war or chance, my fourth year

* Forty-third Annual Meeting, New York, June 23, 1961.

medical quarter was spent that fall under the rack of these two tireless workers. As their chosen scut-boy, one was compelled to work late into the night, hovering over bottles of urine and blood, in tasks which then seemed far beyond the call of duty. But there were compensations: contact with their probing minds over many hours resulted in an approach to clinical medicine which has been a lasting joy; and later at the Massachusetts General Hospital, calcium, phosphorus, bones and urine became integral components of my life blood.

Skipping now some years of what I hope was scientific anabolism, a series of happenings occurred that might well justify Ripley's statement that truth is stranger than fiction. In 1935 a colleague requested consultation on a young man who had developed severe and progressive hypertension immediately after a normal appendix had been removed for abdominal pain. Though we thought the patient did *not* harbor an adrenal medullary tumor, air injection about the kidneys was believed to show a mass; and consequently both kidney areas were exposed simultaneously, as was the custom in those days. The explorer of the suspected left side was the faster operator and the supposed tumor proved to be mythical. The surgeon operating on the right had nearly completed his exploration when the intern holding a retractor became fatigued and permitted the kidney to slide into the operative wound. A yellowish mass was seen in the kidney and believed to be malignant. The surgeon philosophically carried out a nephrectomy and sighed that the operation had not been entirely in vain after all. To my utter amazement, the patient got better and better; within two weeks the blood pressure had become normal, the heart had compensated, vision improved and indeed the patient's blood pressure remains quite normal to this day. Had *not* the radiologists made a wrong diagnosis, the operation *not* been performed *on both sides*, the intern *not* have been up late the night before on some occupation, good or bad, resulting in fatigue, and had the surgeon *not* been wrong in his diagnosis of malignancy (for it proved to be an infarct which would undoubtedly have been left there), that young man would have long since perished.

This stranger than fiction tale does not end here, however. Some twelve years later, because his usual doctor was out of town, I was asked to see a practitioner of medicine whose story was almost identical to that of the boy's just told. He had been explored at Johns Hopkins two months before and appendicitis *not* found. Hypertension, steadily progressive, ensued; and he was now nearly blind and in a state of heart failure. He was told the tale of the boy, and immediately took heart, demanding that his right kidney be removed, for, as he said, "I'm a gone goose if that isn't what's the matter." It took some argument to persuade the urologist to remove the kidney, since the pyelogram was quite normal; but you can imagine our delight to

find the identical lesion present. The practitioner, too, continues to have a normal blood pressure. On the basis of these 2 patients, Dr. Morgan Berthrong investigated the whole problem of renal infarction (these are common findings at post mortem without hypertension) and conclusively showed that atrophy was present surrounding the infarcted zones in our 2 patients and not in 100 other patients with infarcts but without hypertension. Thus a boost was provided to the study of hypertension in man induced by unilateral renal ischemia and its possible cure, a subject which had fallen into severe disrepute because so few nephrectomies had been followed by improvement. But perhaps the strangest and most fictional part of the whole series of incidents was that both surgeons told their patients that the removed appendices were not diseased—which must be rare in the annals of modern surgery since our cutting confreres sported away from the barbers.

Again passing over some years, it became required of me to deliver the Guiteras Lecture before the American Urological Association, owing to the kindness of that Association's then President, my old tennis partner, Dr. J. A. C. Colston. His assigned topic was "The Etiology of Kidney Stones," a subject then beginning to be discussed by their group, rather than mechanical methods for removal of stones, which had been their concern for centuries.

It was a pretty dilemma, and a way out of it seemed to be to stress certain analogies between the behavior of skeletal tissues when they calcify and the probable mechanisms of calculus formation. The more one probed into these matters, the more convincing became the parallelism between *cartilage* calcification, especially, and calcific stone formation. I say cartilage in particular because here we believe that the crystal deposition takes place freely exposed to extracellular water, in distinction to bone where osteoid is almost certainly protected by a membrane with a chemical gradient—be this membrane an anatomic or physico-chemical partition. My colleagues Rubin and Levine had showed that *all forms* of biologic calcification in man have organic matrices with very similar histochemical properties, in that they are metachromatic to toluidine blue and react positively with the Hotchkiss stain. For seventy-five years histologists had told us that one never sees mineral resorption from the skeleton without coincident disappearance of its matrix or osteoid; and, in that Guiteras Lecture, the hypothesis was advanced that enzymatically dissolved skeletal matrix traversed the blood, appeared in the urine and provided the potential matrix for renal calculi. One is far from sure now that this thesis may not be correct in principle.

The next steps stemmed from observations first made by Robison and extensively followed up by the Hopkins pediatric group, including How-

land, Kramer, Park, Wilkins and many others. These consisted essentially of three basic facts:

1. That cartilage in a growing animal will be rachitic, *i.e.*, uncalcified, if there is significant lowering of either calcium or phosphorus concentration (or both) in that animal's serum.

2. That healing of the rickets will occur *in vivo* if the Ca×P "product" is elevated by any means—even including starvation—which raises the serum phosphorus level by way of the protoplasmic breakdown.

3. That the rachitic cartilage will calcify *in vitro* if placed in solutions having concentrations of calcium and phosphorus similar to those required for *in vivo* crystal deposition. In simple solutions the reliability of this calcification *in vitro* is very great to those experienced in the technique of the procedure.

A fourth piece of information was the observation of Jackson and Park, amply substantiated by Follis and others, that a high proportion of patients with chronic renal insufficiency had histologic rickets, despite concentrations of calcium and phosphorus in their sera far too high for rickets to develop in other circumstances.

Yendt and his colleagues followed this up by finding that sera of uremic patients, or its ultrafiltrate, would often fail to calcify rachitic cartilage *in vitro*, despite extraordinarily high Ca×P products. Additions of urea, magnesium and potassium to normal "calcifying" sera in quantities compatible with life, did not prevent *in vitro* calcification; and we pondered on what it was about uremic sera that either acted as a matrix blocker, as a crystal poison, or kept calcium and phosphorus in such a state that their proclivity to crystallize in the matrix was lost. Did something normally emerge from serum into urine with these same properties and, if so, was it what keeps all of us from having a continuous rain of urinary calculi? It had long been a puzzle, and still is, as to how such fabulously high concentrations of calcium and phosphorus can remain in urine without precipitation.

It was this series of musing which led to a request of Dr. William Thomas that he see what would happen to rachitic cartilage if placed overnight in sterile urine which had been brought to pH 7.4 and specific gravity 1.011. Dr. Thomas's reluctance to follow such a silly pursuit may be imagined; disenchantment with an idea could not have been more eloquently exhibited by an army mule. But to his and my amazement, not only did the urine fail to calcify the cartilage but actually appeared to demineralize that portion already calcified to some extent and the bone as well. When this happened over and over again, he turned to the urine of stone formers; and, using exactly the same technique, found that, in the first 8 urines tried, all of these vigorously calcified the cartilage. It should be stated here and now that there is not absolute invariability to these tests, *i.e.*, there are occa-

sional persons who pass urine which calcifies cartilage who have never had a urinary symptom; and sometimes a person with a resident calcific calculus will have a non-calcifying urine. Having been reared in an atmosphere where much emphasis was placed on sin and the devil, for brevity's sake we classified urines which calcify as "evil," and those which do not as "good."

Amusing information has been derived from our somewhat muddled attempts to play with these urines and to change them from good to evil and vice versa. It has been found that dialysis does not alter the good or evil propensity, *i.e.*, the dialysate, when restored by evaporation to its original specific gravity, remains good if the original urine was good, and evil, if that had been the urine's previous bent. *Thus far*, only one reasonably *practical* fact has emerged—a diametric opposite of what might have been anticipated. If one feeds to a recurrent stone-former a considerable quantity of added phosphorus, stone formation will almost invariably cease. This has been shown by a series of patients, some of whom have gone now for three years without new calculus formation; and the average number of stones passed or removed in the first 17 patients of this series was 11 per patient per year over the previous five-year period. Exceedingly painstaking studies by Dr. Purisch on 2 of these patients while on the Metabolism Ward, with urines partitioned throughout the twenty-four hours, have shown that the cartilage tells us that their urines are thoroughly evil on a low phosphorus diet, fairly evil on ordinary phosphorus diets and quite good on high phosphorus diets. Yet, addition of equivalent amounts of phosphorus to the urine of these persons after its passage, though it forms no precipitate, will not induce reversion from the evil to the good state.

We may turn now to the most bizarre aspect of our delvings into the origin and prevention of renal calculi. The fact that stones can grow and solidify in the stream of a fluid current, reminded one of the great discovery of Portland—the combination of materials which form concrete and permit its hardening even under sea water. Portland's finding made possible the jetties and breakwaters for her rugged coasts, without which England could hardly have become mistress of the seas. Cement, of course, has no organic matrix; and heat is required and furnished chemically in the hydration process of its crystal growth. However, perhaps a site on the kidney stone's matrix provides a nidus for crystal initiation, and crystal growth up to a point proceeds in similar fashion to that of concrete. At least, a clue to what prevents concrete from setting, might furnish a means of attack for calculus prevention. To our astonishment it was found that physicists know little or nothing about the actual basic mechanism of concrete hardening. It was of considerable interest also to learn that urine has long been known to inhibit the setting of concrete. We decided, therefore, to see if concrete could distinguish for us a good from an evil urine; and preliminary investi-

gations in this regard were carried out by Russell Morris, Jr., a 12-year-old investigative collaborator, in his cellar during spare time from his school work. When it appeared from young Morris's little blocks of concrete that good and evil urine might indeed reflect a difference in the quality of the product, more elaborate methods of testing were sought. We were fortunate in enlisting the aid of Dr. P. Kenerick Maher, physical chemist, and in charge of the basic cement research of W. R. Grace & Company. Dr. Maher was given 10 small sealed frozen tubes of each of 3 urines, one good, one naturally evil, and the third made evil by chemical manipulation in the laboratory. Using x-ray diffraction of whole pure cement as it was setting, Dr. Maher found distinct and uniform differences between good and bad urines. He then took a single component of the four which go into the purest cement (they will all harden individually with water but won't make nearly as good cement)—namely, tricalcium aluminate, the most important of the components in speeding up cement setting and in the hydration of which a fourth calcium molecule and one of water are accepted. Preliminary patterns with this material again showed a consistent, readily distinguishable difference between the good and evil urines; and most interesting, the curve for water lay right in between the curve for good urine and that for bad. So, weirdly, it may turn out that we will be using cement as a guide to tell us who will and who will not likely have a kidney stone, or whether we are helping or hurting a patient with a given kind of treatment. How crazy can you get in the pursuit of clinical investigation?

For me this has been a most exciting year and I hope one of growth spurt for the Society in its late adolescence. To cap the climax, nothing could have given me more pleasure than to be presiding when the Society's most coveted award was presented to Lawson Wilkins, close friend and fellow traveler along endocrine pathways. The association with him at our Wednesday afternoon sessions for twenty-five years has provided me undoubtedly my best instruction and happiest scientific moments.

I should be remiss if, before closing, I did not express to you my deeply felt appreciation for the honor and privilege of being your President. But I am humbly aware that such encomiums from scientific and academic societies, though bestowed upon an individual, are often in reality an expression of approbation to the colleagues, Fellows and students who have been his co-workers. With warm sentiment my gratitude goes out to those who, through the years, have really done my work, guided my hands and constantly educated me with youthful ideas and elegant criticism. To them—and it is a pleasure to see not a few in this audience—from the bottom of my heart I say thank you.

It is now time to turn over the reins of this organization to an old friend and fellow worker in these vineyards, a man of vigor, originality and integrity—Ted Astwood. The Society will be in good hands.

PURIFICATION OF PITUITARY GONADOTROPIN FROM URINE OF POSTMENOPAUSAL WOMEN*

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ABSTRACT

Human pituitary gonadotropin (HPG) from the urine of postmenopausal women was purified in three stages. After preliminary filtration of the urine with celite, HPG was adsorbed by kaolin, eluted with ammonia and precipitated by 66 per cent acetone. HPG was extracted from the precipitate by 70 per cent ethanol containing 10 per cent ammonium acetate and was prepared in a dry form by precipitation with 90 per cent ethanol. Further impurities were removed by adsorption onto diethylaminoethyl cellulose in 0.05*M* phosphate buffer. The mean purification achieved was 35-fold, with retention of 56 per cent of the original activity. No alteration in the proportions of follicle-stimulating and luteinizing activity of the purified product, as compared with the crude concentrate, was detected. The purified product was 86 times stronger than the unofficial standard HMG-20A, also prepared from postmenopausal urine.

WE RECENTLY described a procedure for the purification of human pituitary gonadotropin (HPG) from male urine (1). The essence of this method is as follows: HPG is removed from the urine by kaolin and, after elution from the kaolin with ammonia, is precipitated by acetone (Fraction A). The HPG in the acetone precipitate is extracted by 10 per cent ammonium acetate in 70 per cent ethanol (Fraction B). Impurities of this extract are removed by diethylaminoethyl cellulose in 0.05*M* phosphate buffer (Fraction C). The yield of HPG activity in Fraction C was about 70 per cent of that originally present in Fraction A, with a purification of about 50-fold. The biologic properties of Fraction C were the same as those of Fraction A, showing that no alteration in the follicle-stimulating and luteinizing activity of HPG had been effected.

The present report is an extension of these studies to postmenopasal urine, which contains four to five times more HPG than does the urine of men.

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MATERIAL AND METHODS

Urine was collected from about 100 postmenopausal women 60 to 100 years of age. The 4 assay methods used to determine the potency and biologic properties of the fractions obtained have been described in full (2).

RESULTS

Preparation of Fraction A. Fraction A was prepared from 1200 liters of postmenopausal urine, as previously described (2). Fraction A is crude HPG and serves as the reference material against which all purified fractions are compared.

Preparation of Fraction B. A total of 60 liters of urine adjusted to pH 4.5 with glacial acetic acid is placed in a large metal separatory funnel; 200 gm. of celite is stirred into the urine, and the celite is allowed to settle through the stopcock into a removable container. When settling has occurred (usually overnight), the container is removed and the celite discarded. Kaolin (300 gm.) suspended in acidified tap water (pH 4.5) is stirred into the urine. The kaolin is allowed to settle overnight through the stopcock, after which it is removed from the funnel. The urine is now discarded, and the separatory funnel is filled with 60 liters of cold tap water containing 30 ml. of glacial acetic acid and 600 gm. of commercial salt (Morton's flake salt). The kaolin is added to the water with stirring and again allowed to settle through the stopcock into the collecting vessel. The next day, the kaolin is removed. Four 50-ml. beakerfuls (44 gm.) of celite are added to the kaolin, and then sufficient distilled water is added to make a total volume of 2 liters. This mixture is stirred, and 500-ml. portions are distributed to each of 4 filtration chambers. The excess of fluid is removed by rapid pressure filtration. The celite-kaolin cake is then removed and mixed in a Waring blendor with 200 ml. of 2*M* ammonium hydroxide, and the elution procedure as described for the elution of Fraction A is carried out (1). The eluate from the 4 chambers is acidified, mixed with acetone and allowed to stand at 5° C. overnight. A half beakerful of celite is then added to the 5400 ml. of 66 per cent acetone and thoroughly mixed. This mixture is poured into a filtration chamber and rapidly filtered. The filter cake is flushed with 200 ml. of acetone, both the 66 per cent acetone filtrate and the acetone wash being discarded. The celite cake containing the 66 per cent acetone precipitate is transferred to a Waring blendor and mixed with 120 ml. of 10 per cent ammonium acetate in 70 per cent ethanol. This mixture is rapidly filtered in a filtration chamber. The filtrate is collected and passed through the chamber again. A fresh portion (120 ml. of 10 per cent ammonium acetate in 70 per cent ethanol) is added to the filtration chamber and passed through the cake. To the combined filtrate (240 ml. of 10 per cent ammonium acetate in 70 per cent ethanol), 2 volumes of absolute alcohol

TABLE 1. CHARACTERISTICS OF FRACTION B: POSTMENOPAUSAL URINE

No.	Solids (mg./liter)	S.A.*
1	3.0	6.1
2	4.2	13.1
3	1.3	5.5
4	3.4	8.0
5	0.9	14.2
6	2.9	8.0
7	1.6	5.8
8	1.8	9.7
9	0.6	26.8
10	2.0	5.9
11	1.6	16.0
12	2.7	11.5
13	1.3	5.0
14	2.2	9.0
15	1.9	13.7
16	1.9	14.5
17	1.6	25.1
18	1.5	12.6
19	4.2	5.1
Mean	2.1	11.3

* S.A. = specific activity, rat units per milligram (R.U./mg.).

TABLE 2. YIELD AND SPECIFIC ACTIVITY OF FRACTION C FROM 1 GM. OF FRACTION B*: POSTMENOPAUSAL URINE

No.	Solids (mg.)	S.A.
1	296.9	23.2
2	282.5	31.2
3	281.5	48.8
4	293.6	46.8
5	294.6	29.6
6	313.7	30.2
Mean	293.8	35.9

* 1 gm. of Fraction B = 476 liters of urine; 1 gm. of Fraction C = 1620 liters of urine.

saturated with ammonium acetate is added. The mixture is placed in a refrigerator overnight. The precipitate that forms is removed by centrifugation and dried with alcohol and ether in the usual fashion.

Table 1 shows 19 consecutive preparations of Fraction B. The mean yield of solid material was 2.1 mg. per liter of urine, with a mean specific activity of 11.3 rat units per milligram.

Preparation of Fraction C. Fraction C was prepared from 1-gm. portions of Fraction B, as previously described (1). Table 2 shows the results of 6 experiments. The data indicate that 70 per cent of the solids present in Fraction B were removed, and the resulting specific activity of Fraction C was about three times greater than that of Fraction B.

Biologic properties of Fractions A, B and C. Table 3 shows the general gonadotropin assays for the 3 fractions, the yield of solids per liter, the purification achieved, and the losses encountered. On the basis of the general gonadotropin assays, Fraction C is 86 times more potent than the unofficial standard HMG-20A, prepared from postmenopausal urine.

TABLE 3. GENERAL GONADOTROPIN ASSAYS: POSTMENOPAUSAL URINE

Fraction	Solids (mg./liter)	No. of assays	Specific activity			Activity per liter		
			R.U./mg.	Mg. of HMG-20A/mg.	Pf.*	R.U.	Mg. of HMG-20A	Per cent
A	38.5	21	0.8	1.9	1	31.2	75	100
B	2.1	29	11.2	26.9	14	23.5	56	75
C	0.6	6	35.9	86.2	44	22.2	53	71

* Pf. = purification.

TABLE 4. FINGERPRINT ASSAYS: POSTMENOPAUSAL URINE

Fraction	No. of assays	Slopes		Dose ratio, O:U*
		Ovary	Uterus	
A	4	119	357	1.8
B	2	149	314	1.9
C	2	132	224	1.7

* O:U = ovary:uterus (ratio of threshold doses).

Fingerprint assays are shown in Table 4. The three important parameters, namely the slopes of the ovarian and uterine curves and the ovarian :uterine dose ratio, are of the same order of magnitude for all 3 preparations. This indicates that no great alteration in the composition of HPG had occurred during purification.

The assays for "follicle-stimulating hormone" (FSH) (ovarian-augmentation activity) are shown in Table 5 in conjunction with augmentation assays for the NIH-FSH-S1 standard. Fraction C is almost as potent as the NIH-FSH-S1 standard. Also shown in Table 5 is the ovarian-weight activity of the 3 fractions, an assay that is similar to the general gonadotropin assays (2).

Ventral-prostate assays for luteinizing-hormone (LH) potency are shown in Table 6. Fraction C is about one third as potent as the NIH-LH-S1 standard.

COMMENT

The purification of HPG from postmenopausal urine was accomplished by methods similar to those detailed previously for male urine. However, the urine of postmenopausal women cannot be handled in exactly the same manner as is urine of normal men. Fraction A of postmenopausal urine contains considerably less solids than does that of men; consequently, its specific activity is 10 times that of Fraction A from the urine of men. Despite this favorable advantage, postmenopausal urine, possibly because of the bacterial content or cellular debris, could not be processed through the automatic filters. To improve flow rates, preliminary filtration of post-

TABLE 5. AUGMENTATION ASSAYS: POSTMENOPAUSAL URINE

Fraction	No. of assays	Ovarian slopes		S.F.†	Ovarian-augmentation activity				Ovarian-weight activity			
		Alone	+HCG*		R.U./mg.	Pf.	R.U./L.	Per cent	R.U./mg.	Pf.	R.U./L.	Per cent
A	2	113	179	3.8	0.21	1	8.1	100	0.16	1	6.2	100
B	2	127	183	4.5	2.7	13	5.7	70	1.7	11	5.7	92
C	2	129	195	5.6	7.8	37	4.8	59	4.4	28	2.6	44
NIH-FSH-S1	3	52	153	51.6	9.2	—	—	—	2.9	—	—	—

* HCG = human chorionic gonadotropin.
† S.F. = sensitivity factor (2).

TABLE 6. VENTRAL-PROSTATE ASSAYS: POSTMENOPAUSAL URINE*

Fraction	No. of assays	S.A.	Pf.	Activity per liter	
				R.U.	Per cent
A	3	0.22	1	8.5	100
B	2	2.3	10	5.0	59
C	2	6.9	32	4.3	51
NIH-LH-S1	5	24.3	—	—	—

* Abbreviations same as in preceding tables.

menopausal urine with celite was required before it could be processed by the same technic worked out for male urine. Although celite removes only inert material and does not affect the FSH and LH content, a strict comparison of yields and activities between the two urines could not be made by using previously published data on male urine. Therefore, in order to obtain a valid comparison, male urine was processed in exactly the same way as described here for postmenopausal urine. Fraction A of male urine already prepared (2) is the exact counterpart of Fraction A from postmenopausal urine. Male Fraction B was prepared exactly as described for postmenopausal Fraction B. This preparation contained 3.8 mg. of solids per liter of urine, 1 gm. being equivalent to 263 liters of urine. Male Fraction C was prepared from 10 gm. of male Fraction B, yielding 1.1 mg. of solids per liter of urine; 1 gm. of male Fraction C was equivalent to 909 liters of urine.

The biologic assays of male Fractions A, B and C are shown in Tables 7–10. Except for the difference in potency, these assays show that the HPG of postmenopausal urine behaves in the same fashion as does that of male urine during the purification procedure. The summation of these similarities is shown in Table 11. Despite the initial difference in the solid content of Fraction A from the two sources of urine, the yield in solids of Fractions B and C, expressed as a percentage of the solids in respective Fractions A, is the same for both postmenopausal and male urine. To obtain an estimation of the losses encountered and the purification achieved, the entries in the tables for these values in all 4 assays were averaged. These means are also

TABLE 7. GENERAL GONADOTROPIN ASSAYS: MALE URINE*

Fraction	Solids (mg./liter)	No. of assays	Specific activity			Activity per liter		
			R.U./mg.	Mg. of HMG-20A/mg.	Pf.	R.U.	Mg. of HMG-20A	Per cent
A	80.7	4	0.089	0.2	1	7.1	17	100
B	3.8	9	1.5	3.6	18	5.7	13.7	80
C	1.1	2	4.0	9.6	48	4.4	10.6	62

* Abbreviations same as in preceding tables.

TABLE 8. FINGERPRINT ASSAYS: MALE URINE*

Fraction	No. of assays	Slopes		Dose ratio, O:U
		Ovary	Uterus	
A	6	111	254	2.0
B	2	104	243	1.8
C	3	100	215	2.1

* Abbreviations same as in preceding tables.

TABLE 9. AUGMENTATION ASSAYS: MALE URINE*

Fraction	No. of assays	Ovarian slopes		S.F.	Ovarian-augmentation activity				Ovarian-weight activity			
		Alone	+HCG		R.U./ mg.	Pf.	R.U./ L.	Per cent	R.U./ mg.	Pf.	R.U./ L.	Per cent
A	2	146	180	3.9	0.022	1	1.8	100	0.016	1	1.3	100
B	2	142	239	3.6	0.39	18	1.5	83	0.27	17	1.0	77
C	3	135	151	4.4	1.2	55	1.3	72	0.73	46	0.8	62
NIH-FSH-S1	3	52	153	51.6	9.2	—	—	—	2.9	—	—	—

* Abbreviations same as in preceding tables.

TABLE 10. VENTRAL-PROSTATE ASSAYS: MALE URINE*

Fraction	No. of assays	S.A.	Pf.	Activity per liter	
				R.U.	Per cent
A	5	0.029	1	2.3	100
B	3	0.41	14	1.6	70
C	3	1.1	38	1.2	52
NIH-LH-S1	5	24.3	—	—	—

* Abbreviations same as in preceding tables.

shown in Table 11. On this basis, the loss of activity during processing was approximately the same for both male and postmenopausal urine, but the purification achieved was somewhat less for postmenopausal urine than for male urine.

Partly purified preparations of HPG from postmenopausal urine have been reported on 5 occasions. These materials are (a) HMG-20A prepared by Dr. Dekanski, (b) HMG-J5 prepared by Dr. Johnsen, (c) Pergonal prepared by Dr. Donini, (d) HMG prepared by Drs. Segaloff and Steelman and (e) I.R.P. (International Reference Preparation) prepared by Dr. Dekanski. The methods of preparation and the properties of these 5 materials are fully described in a recent monograph (3). Despite the information given in this monograph, it is not yet possible to compare these 5 preparations qualitatively with Fraction C, because different standards for FSH and LH activity were used. Assays of these materials in terms of the NIH standards for FSH and LH are valid with the ovarian-augmentation method and the ventral-prostate method, respectively, and further work is

TABLE 11. COMPARISON OF YIELD AND PURIFICATION OF HPG FROM MALE AND POSTMENOPAUSAL URINE

Fraction	Yield, solids			
	Men		Postmenopausal women	
	Mg./L.	Per cent	Mg./L.	Per cent
A	80.7	100	38.5	100
B	3.8	4.7	2.1	5.5
C	1.1	1.4	0.62	1.6
	Mean yield, activity per liter			
	Men		Postmenopausal women	
A	100		100	
B	77		74	
C	62		56	
	Mean purification			
	Men		Postmenopausal women	
A	1		1	
B	17		12	
C	47		35	

needed to characterize qualitatively the 6 different HPG preparations from postmenopausal urine.

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A GONADOTROPIN-INHIBITING SUBSTANCE IN THE URINE OF NORMAL YOUNG CHILDREN

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ABSTRACT

The presence of a gonadotropin-inhibiting factor in the urine of normal young children is reported. Urinary extracts were tested by the mouse uterine weight response, assayed against purified HCG. This factor is consistently observed in the urine of children under the age of 6 years, and specifically inhibits the action of gonadotropin. It exercises no inhibitory effect on estrogen.

IN A preliminary report published in 1960 (1) we described the presence of a gonadotropin-inhibiting substance in the urine of normal children. This substance was consistently observed in the urine of children under the age of 6 years and was less uniformly present in older children. The inhibiting substance was equally demonstrable in the urine of both sexes within this age group. In the original report, the inhibiting substance extracted from the urine was assayed against the gonadotropic activity of pooled human adult urine. The present report is an extension of the original study and was designed to establish the following theses: 1) that the inhibition of gonadotropic activity induced by extracts of children's urine was not an artifact, and 2) that the substance in question exercised a specific inhibitory effect on gonadotropic activity.

METHOD

In the present studies, purified human chorionic gonadotropin (HCG)[1] was used to obtain a more consistent and better standardized mouse uterine weight response. The HCG was assayed in 18-day-old weanling female mice. The total dose of HCG per mouse in the present series was 0.3 I.U., and of estrone[2] 0.1 μg. The amount of hormone employed was that which resulted in a uterine weight varying between 20 and 25 mg.

The inhibiting substance was extracted from children's urine by the technique ordinarily used for the extraction of gonadotropin from urine. The method employed was a

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[1] U.S.P. reference standard, generously supplied by the U. S. Pharmacopoeial Convention Inc.

[2] The estrone was generously supplied by Dr. Kenneth Thompson of Organon, Inc.

modification (2) of the technique of Klinefelter, Albright and Griswold (3), involving the use of the kaolin absorption method of Bradbury, Brown and Brown (4). The final dry residue from each liter of pooled urine was dissolved in 2.5 ml. of physiologic sodium chloride. After centrifuging, the supernatants were collected and pooled. The pH of the urinary extract varied from 4.5 to 5.0. For the present study, only the urine of children under the age of 6 years was used.

Extracts from 25 to 400 ml. of urine were assayed against 0.3 I.U. of HCG in one series of experiments, and extracts from 400 ml. of urine were tested against 0.1 μg. of estrone in another series. Both the hormone and the urinary extract were injected subcutaneously five times over a three-day period. When HCG was used, each injection consisted of 0.2 ml. of hormone and an equal amount of either physiologic saline or urinary extract. When estrone was employed, each injection contained 0.1 ml. of hormone (in an olive oil suspension) plus 0.2 ml. of physiologic saline or urinary extract. Each substance was injected at a different site. Eighteen hours after the fifth injection the mice were sacrificed (ether anesthesia). The uteri were carefully dissected, dried by blotting on Whatman No. 1 paper, and weighed on an analytical torsion balance. The average uterine weights were then compared.

RESULTS

In Table 1 are listed the results obtained when extracts from varying amounts of the urine of normal children were assayed against a given amount of human chorionic gonadotropin. The extracts from 400 and 300 ml. of urine had a distinct inhibitory effect on the gonadotropic activity of the HCG, although inhibition was not complete. In these 2 groups (D and E), 75 per cent or more of the mice demonstrated such inhibition. With lesser concentrations of urine the inhibitory effect became less pronounced, and when the extracts from amounts of urine less than 100 ml. were tested against the HCG, the results were equivocal or negative.

Statistical evaluation of the individual groups of animals was undertaken to establish whether the responses of the different groups were significantly different from each other. Table 2 lists the number of mice in each group, with the mean and standard distribution of their uterine weights. In the 2 control groups (A and B) the uterine weights were within a relatively nar-

TABLE 1. EFFECT OF EXTRACT OF URINE FROM CHILDREN UNDER THE AGE OF 6 YEARS ON THE GONADOTROPIC ACTIVITY OF HUMAN CHORIONIC GONADOTROPIN
(total dose, 0.3 I.U. per mouse)

Experiment	Group	No. of mice	No. that died	Avge. uterine wt. (mg.)	Per cent of mice with uterine wts. of 15 mg. or more	Per cent of mice with uterine wts. of less than 15 mg.	Per cent of mice with gain in body wt.	Per cent of mice with loss or no change in body wt.
Saline control	A	25	1	7.5	0	100	48	52
Urine, 400 ml.	B	12	0	7.5	0	100	33.3	66.7
HCG, 0.3 I.U.	C	45	0	25.4	75.6	24.4	75.5	24.5
HCG +400 ml. urine	D	22	1	13.4	22.7	77.3	0	100
HCG +300 ml. urine	E	16	0	12.0	18.8	81.2	25	75
HCG +200 ml. urine	F	16	0	17.5	50.0	50.0	31.3	68.7
HCG +100 ml. urine	G	16	0	15.8	50.0	50.0	37.5	62.5
HCG +50 ml. urine	H	16	0	23.0	43.7	56.3	81.0	19.0
HCG +25 ml. urine	I	16	0	22.2	50.0	50.0	87.5	12.5

TABLE 2

Group	Experiment	No. of mice	Range of uterine wts.	Mean wt. (χ)	Standard deviation (σ)
A	Saline control	25	4.0–10.0	7.26	± 1.66
B	Urine, 400 ml.	12	4.3–10.4	7.43	± 1.94
C	HCG, 0.3 I.U.	45	6.8–66.5	25.31	±12.8
D	HCG+400 ml. urine	22	7.7–32.2	13.39	± 4.95
E	HCG+300 ml. urine	16	6.0–36.0	11.96	± 6.90
F	HCG+200 ml. urine	16	7.7–43.4	17.52	± 9.59
G	HCG+100 ml. urine	16	5.6–28.3	15.81	± 6.61
H	HCG+50 ml. urine	16	6.8–49.9	22.95	±15.0
I	HCG+25 ml. urine	16	5.0–59.5	22.17	±14.5

row range. Thirty of the 38 mice receiving maximal amounts of urinary extract plus HCG (Groups D and E) had uterine weights less than 15 mg. One animal in each group showed a sharp response to the HCG (32.2 and 36.0 mg.), resulting in a large increase in the variance calculated for these 2 groups. With smaller quantities of urinary extract, the response became increasingly variable, so that extracts obtained from 25 and 50 ml. of urine (Groups H and I) when assayed against the HCG yielded results approximating those obtained with the latter alone (Group C).

A comparison of these groups (Table 3) revealed that, despite the wide variations in Groups C and F through I, certain conclusions could be drawn:

TABLE 3

Groups	Difference in means $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$	Probability of chance occurrence (P)
A–B	0.26	0.87
A–C	12.80	<.001
B–C	11.31	<.001
A–D	5.57	<.001
B–D	5.01	<.001
A–E	2.67	.007
A–F	4.24	<.001
A–G	5.09	<.001
A–H	4.16	<.001
A–I	4.08	<.001
C–D	5.47	<.001
C–E	5.17	<.001
C–F	2.56	.009
C–G	3.77	<.001
C–H	0.56	.576
C–I	0.77	.440
E–G	1.61	.110
E–H	2.65	.008
E–I	2.54	.011

TABLE 4. EFFECT OF EXTRACT OF URINE FROM CHILDREN UNDER THE AGE OF 6 YEARS ON THE INCREASE IN MOUSE UTERINE WEIGHT INDUCED BY ESTRONE*

Experiment	No. of mice	No. that died	Avge. uterine wt. (mg.)	Per cent of mice with uterine wts. of 15 mg. or more	Per cent of mice with uterine wts. of less than 15 mg.	Per cent of mice with gain in body wt.	Per cent of mice with loss or no change in body wt.
Physiologic saline	12	0	7.2	0	100	91.7	8.3
Oil	12	0	8.2	0	100	91.7	8.3
Urine, 400 ml.	6	0	7.9	0	100	83.3	16.7
Estrone-in-oil, 0.1 μg.	14	0	23.6	100	0	93.8	6.2
Estrone-in-oil, 0.1 μg. +400 ml. urine	16	0	25.3	100	0	50	50

* *Statistical data.* Estrone, 0.1 μg.: Standard Deviation = 5.15; $t = .83$.
Estrone + Urine: Standard Deviation = 6.10; $P = >.7$.

1. The average values in the control groups (A and B) were significantly different from those in the group treated with HCG alone (C).
2. Each of the groups receiving urinary extract plus HCG (D through I) showed a significantly increased uterine weight response compared to the control groups (A and B).
3. The average uterine weights in the 4 groups (D through G) receiving the highest concentrations of urinary extract plus HCG were significantly less than in the groups treated with HCG alone (C).
4. There was no difference between the uterine weights in the 2 groups (H and I) receiving the smallest concentrations of urinary extract plus HCG and the weights in the group receiving HCG alone (C).

On the basis of the experimental data it may be concluded that mice receiving HCG and extract obtained from 100 to 400 ml. of urine showed a increase in uterine weight which was *significantly less* than would be expected in mice receiving this amount of HCG alone.

In an attempt to determine whether the urinary extract exercised a specific inhibitory effect against HCG, such extracts were also assayed against estrone. In Table 4 these results are recorded. It is evident that the urinary extract exercises *no* inhibitory effect on estrone.

DISCUSSION

The data suggest that an extract from the urine of normal young children contains a substance that exercises an inhibitory effect on gonadotropin. The question arises as to the possible role of toxicity of the urinary extracts in this phenomenon, as indicated by the greater incidence of weight loss in the urine-treated animals. This is a possibility that we cannot exclude com-

pletely. However, inhibition occurred with extracts from 100 and 200 ml. of urine when the incidence of failure of the mice to gain weight was not much greater than that observed in the saline-treated controls (62.5 per cent and 68.7 per cent as against 52 per cent, respectively). In addition, 50 per cent of the mice receiving urinary extract plus estrone failed to show any gain in weight despite an average uterine weight within the same range as that observed in the animals treated with estrone alone. In the latter group only 6 per cent of the animals failed to gain weight.

Our data appear to support the contention that the observed inhibition represents a response to a specific inhibitor rather than a toxic effect of the urinary extract.

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DETERMINATION OF IODINE COMPOUNDS IN SERUM. I. SERUM THYROXINE IN THE PRESENCE OF SOME IODINE CONTAMINANTS

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ABSTRACT

A column method utilizing the anion-exchange resin Dowex-1 is described for the determination of thyroxine in serum. Values comparable to those for butanol-extractable iodine were obtained, and additions of inorganic iodine or iodotyrosines did not affect the reliability. Analyses of sera containing *in vitro* additions of organic iodine compounds which interfere with the PBI and in most cases the BEI, gave results which suggested the possible usefulness of the column method. Furthermore, the pattern of thyroxine elution with the column method, as determined by the iodine content of 3 successive 10-ml. fractions, was helpful in predicting the validity or nonvalidity of the result. When the BEI and column techniques were applied to cases in which elevated serum PBI values did not correlate with the clinical findings, there was some evidence for better specificity of the column method. Neither of the methods was helpful in most cases involving both inorganic iodine and PBI values in excess of 25 μg. per 100 ml.

THE presence in serum of organic iodine compounds used for radiographic and therapeutic purposes precludes determination of thyroid hormones as presently measured by the protein-bound iodine (PBI) and butanol-extractable iodine (BEI) methods. The length of time for which these compounds can interfere ranges from a few days to many years, thus depriving the physician of two of the most valuable laboratory tests of thyroid function. Furthermore, unless the result is very high, one cannot be sure whether interference is present or not.

In view of the work of numerous investigators (1–7) demonstrating the usefulness of ion-exchange resins for separation of serum iodo-amino acids and inorganic iodine, it was decided to study this approach for separation of thyroxine (T_4) from other organic iodine compounds with the hope that a test could be evolved possessing greater accuracy for thyroxine than either the PBI or BEI test.

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METHODS

Preparation of the columns. The resin used was Dowex-1, X-2, 200–400 mesh, analytical grade, in the Cl^- form. A column 4 cm. high $\times 1\frac{1}{2}$ cm. in diameter was washed first with 50 per cent acetic acid and then adjusted to pH 4.0 by washing with 0.2M acetate buffer.

Column method for determination of thyroxine in serum. Serum was adjusted to pH 4.0 by addition of 0.2 *M* acetate buffer, pH 2.6, (usually 0.5 ml. of buffer required per ml. of serum). When normal levels of thyroxine were anticipated, a 3.0-ml. aliquot of serum was sufficient. Quantitative transfer to the column was followed by washes with 25 ml. each of 0.2 *M* acetate buffers of pH 4.0 and 3.0, and 50 ml. of acetic acid, pH 2.2 (prepared by diluting glacial acetic acid with water). Thyroxine was then eluted from the column at pH 1.4 (approximately 50 per cent acetic acid) and the eluate collected in three successive 10-ml. fractions. 3,5,3′-Triiodothyronine is also eluted at pH 1.4, as indicated by Galton and Pitt-Rivers (3) and confirmed by us. Suitable aliquots (generally 3.0 ml.) of each fraction were transferred to 16×100 mm. Pyrex tubes containing precipitated and washed $Zn(OH)_2$ (5.0 ml. of 0.05 *N* NaOH plus 1.0 ml. of 5 per cent $ZnSO_4$) and the contents evaporated to dryness. To the dried residue was added 0.5 ml. of 2 *N* Na_2CO_3 containing 1 per cent $KClO_3$, and 0.5 ml. of a 4 per cent solution of purified gamma globulin (bovine, fraction II, Pentex Corp., Kankakee, Illinois). After bringing to dryness again by heat, the tubes were placed in an incinerator and the contents ashed at approximately 610° C. for forty-five minutes. Determination of iodide in the ash was made by use of the ceric-arsenious acid reaction (8, 9).

PBI and BEI determinations. PBI determinations were made by the alkaline incineration method of Barker *et al.* (9) with certain minor modifications of technique which included a smaller sample (0.5 ml.) and a shorter incineration time (610° C. for forty-five minutes). The method of Man *et al.* (10) was used for BEI determinations, a major modification being an alkaline incineration of the residue remaining after evaporation of the Blau's washed butanol extract with glycine added as carrier.

Radioactive materials. I^{131}-labeled thyroxine (Abbott Laboratories) was checked for purity at the time of use by descending chromatography in a 2-butanol-4% NH_4OH (3:1) system. These studies were carried out on inch-wide strips (Whatman #3) which, after drying, were scanned with a paper strip scanner (Scanogram II, Atomic Accessories) and the percentage of radioactivity due to thyroxine determined by planimetry.

RESULTS

Recovery of radioactive thyroxine from serum by the column method

I^{131}-labeled T_4 was added to serum which was then incubated for two hours at 37° C. The amount of T_4 added was 1.2 μg. per 100 ml. of serum. After adjustment of 3.0 ml. of serum to pH 4.0 with acetate buffer, it was transferred to a column (also equilibrated at pH 4.0 as described under Method) and fractions collected as indicated in Table 1. The experiment shown was typical of 6 such studies. Of the total radioactive T_4 transferred onto the column, 6.5 per cent was not absorbed. The pH-3.0 wash did not remove any of the T_4, and 50 ml. of pH-2.2 wash removed less than 1 per cent. In agreement with Galton and Pitt-Rivers' findings (3), T_4 was eluted in a relatively small volume at pH 1.4. More than 80 per cent was recov-

TABLE 1. RECOVERY OF RADIOACTIVE THYROXINE FROM SERUM

Fraction	Volume (ml.)	pH	Radioactivity counts/min.	Per cent of total
1	29	4.0	47,880	6.5
2	20	3.0	0	0.0
3	50	2.2	6,430	0.9
4	10.0	1.4	634,400	86.0
5	10.2	1.4	44,221	6.0
6	10.2	1.4	2,264	0.3
7	9.4	1.4	1,438	0.2
Total eluted material			736,633	99.9
Total radioactive T_4 used			737,000*	100.0

* This value represents radioactivity due to thyroxine. A correction for impurities was made based on paper chromatography which revealed a radiopurity of 95%.

ered in 10.0 ml. of eluate and over 90 per cent in 20 ml. Expressed as the percentage of eluted radioactivity, 94 per cent of the thyroxine was present in the first 10-ml. fraction. This pattern of elution was found to exist at levels of thyroxine iodine as high as 19.0 μg. per 100 ml. (highest level studied), and was of help in evaluating the validity of a column analysis from the viewpoint of separation of thyroxine from other organic iodine compounds.

Reproducibility of the column method was investigated by analyzing a pooled serum on 6 separate columns. For this study a serum with a PBI value of 4.8 μg. per 100 ml. and a BEI value of 4.0 μg. per 100 ml. was used. The mean and standard deviation obtained were 4.0 and 0.24 μg. per 100 ml., respectively. This reproducibility is similar to that usually obtained for the PBI or BEI methods (10, 11).

Comparison of column, PBI and BEI results on random sera

A group of random sera was collected for BEI and column analyses solely on the basis of covering a wide range of PBI values. Results are shown in Table 2 and illustrate a close correlation between the BEI and column results; statistical analysis of the data by the *t* test revealed no significant difference ($P > 0.1$).

Effect of added iodide, iodotyrosines and organic iodine contaminants on the reliability of the column, BEI and PBI methods

The column method was tested on sera containing elevated concentrations of inorganic iodine or iodotyrosines—compounds interfering with the PBI determination but not with the BEI. No interference was observed (Table 3) at a level of contamination of 1000 μg. per 100 ml. of iodide or 6.6 μg. per 100 ml. of iodine as iodotyrosines (mono- and diiodotyrosine).

The effect of various organic iodine compounds on the reliability of the

TABLE 2. COMPARISON OF COLUMN METHOD WITH PBI AND BEI METHODS ON RANDOM SERA*

Sample	Serum level (μg. iodine/100 ml.)		
	PBI method	BEI method	Column method (sum of fractions 1 and 2)
1	2.1	1.2	1.0
2	3.1	1.2	1.2
3	3.7	3.2	2.8
4	4.3	2.7	3.3
5	5.0	3.5	4.6
6	5.7	5.1	4.3
7	5.9	1.4	1.1
8	6.9	6.6	6.4
9	8.2	7.3	7.4
10	9.4	4.5	4.8
11	11.1	9.1	7.3
12	13.0	11.6	9.8
13	14.3	12.6	14.0
14	15.7	13.5	13.8
15	17.1	13.6	12.6
16	23	20	15.7
Mean	*9.3*	*7.3*	*6.9*

* The PBI and BEI values are averages of duplicate determinations; the column values are for single analyses with duplicate iodine determinations on the fractions.

PBI, BEI and column techniques is shown in Table 4. Compounds were added to a control serum which was then incubated at 37° C. for two hours; the final concentration of each compound was approximately 1000 μg. per 100 ml. expressed as iodine. PBI and BEI values were obtained by averaging the results of 2 analyses performed in duplicate on separately prepared serum samples. A single column analysis was performed on each of the separately prepared serum samples, but duplicate iodine analyses were made on each fraction.

PBI results were in every case unreliable, and in all but 2 instances the values were grossly elevated (>20 μg. per 100 ml.), so that confusion with

TABLE 3. EFFECT OF IODOTYROSINES AND IODIDE ON RELIABILITY OF PBI, BEI AND COLUMN METHODS*

Additions to serum	Serum level (μg. iodine/100 ml.)				
	PBI method	BEI method	Column method (fraction)		
			1	2	3
None	4.8	3.9	3.5	0.6	0.1
MIT+DIT†	10.8	4.0	3.3	0.6	0.2
KI‡	7.1–9.9	3.8	3.1	0.9	0.1

* PBI and BEI values are averages of 4 determinations; column values are averages of duplicate analyses.

† Monoiodotyrosine and diiodotyrosine present at a concentration of 3.3 μg. per 100 ml. (as iodine) each.

‡ KI present at a concentration of 1000 μg. per 100 ml. (as iodine); precipitate washed four times with distilled water.

physiologic levels of T_4 would be unlikely. The two exceptions (after addition of Miokon and Organidin) are noteworthy in view of the possible misleading nature of the results from a diagnostic point of view. This is especially true of Miokon, addition of which resulted in variable nonreproducible values which probably reflect a loose binding to the serum proteins. This concept is supported by an elevated inorganic iodine level (25–500 μg. per 100 ml.) in a screening test for both Miokon and Organidin. This screening test measures nonprecipitable compounds which catalyze the ceric-arseni-

TABLE 4. EFFECT OF *in vitro* ADDITIONS OF ORGANIC IODINE COMPOUNDS ON RELIABILITY OF PBI, BEI AND COLUMN METHODS*

Iodine compound added to serum		Serum level (μg. iodine/100 ml.)				
Chemical name	Common	PBI method	BEI method	Column method (fraction)		
				1	2	3
None	None	4.8	3.9	3.4	0.5	0.0
N,N'-adipyl-bis-3-amino-2,4,6-triiodobenzoic acid	Cholografin	>25.0	7.5; 20.0	3.4	0.6	0.0
3,5-Diiodo-4-pyridone-N-acetic acid, n-propyl ester	Dionosil	>25.0	>25.0	5.7	2.6	>25.0
o-Iodohippuric acid	Hippuran	>25.0	>25.0	>25.0	0.6	0.0
5-Iodo-2-thiouracil	Itrumil	22.0	17.0	>25.0	4.7	2.0
3,5-Dipropionylamino-2,4,6-triiodobenzoic acid	Miokon	7.0; 12.9†	4.3	9.1	12.8	13.1
1-Methyl-3,5-diiodo-4-pyridone-2,6-dicarboxylic acid	Neo-Iopax	23.0	4.4	3.0	0.6	0.0
3-(3-Butylamino-2,4,6-triiodophenyl)-2-ethyl acrylic acid	Orabilex	>25.0†	>25.0	>25.0	15.2	2.3
Iodopropylidene glycerol	Organidin	8.7†	>25.0	3.4	0.4	0.0
3-(4-Hydroxy-3,5-diiodophenyl)-2 phenylpropionic acid	Priodax	>25.0	12.5;>25.0	3.5	4.5	6.2
3-Acetylamino-2,4,6-triiodobenzoic acid+polyvinyl pyrrolidone	Salpix	>25.0	4.7; 7.1	3.3	0.8	1.6
Iodomethane sulfonic acid	Skiodan	>25.0	4.1	3.3	0.5	0.0
3-(3-Amino-2,4,6-triiodophenyl)-2-ethyl propionic acid	Telepaque	>25.0	>25.0	>25.0	13.3	8.9
3-(3-Hydroxy-2,4,6-triiodophenyl)-2-ethyl propionic acid	Teridax	>25.0	>25.0	>25.0	>25.0	>25.0
3-Acetylamino-2,4,6-triiodobenzoic acid	Urokon	>25.0	5.5; 6.4	3.5	0.6	0.2

* All values are averages of 2 analyses on separately prepared samples (PBI and BEI in duplicate; column analyses, single determinations).
† Elevated inorganic iodine levels in screening test (25–500 μg. per 100 ml.).

ous acid oxidation-reduction; organic iodine compounds fall into this category.

BEI results were reliable in the presence of Miokon, Neo-Iopax and Skiodan, were variably elevated in the presence of Urokon, Salpix and Cholografin, and were grossly elevated for the remaining 8 compounds studied. The multiple results shown for several of the compounds are averages of duplicate determinations on separately prepared samples. The widely divergent values are probably due to nonquantitative separation of thyroxine from the contaminant. Since 1000 μg. of contaminant was added per 100 ml. of serum, small differences in the percentage of contaminant extracted or removed would be significant in relation to the true value of 3.9 μg. per 100 ml.

Results of the column analyses were calculated as μg. per 100 ml. of

serum, based on the iodine content of each of three 10-ml. fractions collected. In evaluating the validity of a particular column analysis it was assumed, on the basis of studies with radioactive T_4 (Table 1), that the first 10-ml. fraction should contain over 90 per cent of the eluted thyroxine. Deviations from this percentage distribution would suggest simultaneous elution of the added iodine compound and the possible invalidity of the thyroxine determination. Values comparable to those for the control serum were obtained in the presence of Cholografin, Neo-Iopax, Organidin, Salpix, Skiodan and Urokon. If the first fraction was considered to be a useful estimate of the thyroxine level, then potentially useful results were also obtained in the presence of Priodax, and possibly Dionosil. In all other instances the pattern of elution clearly demonstrated the presence of non-thyroxine iodine. At lower levels of contamination, however, the pattern may not prove as helpful in instances in which the organic iodine compound is also found mainly in the first fraction (*e.g.*, Hippuran, Itrumil, Orabilex and Telepaque).

In order to assess the possible usefulness of the column technique from a clinical viewpoint, patients' sera were selected on the basis of containing concentrations of PBI which were either grossly elevated (a value in excess of 25 μg. per 100 ml. arbitrarily was assumed to represent artifactually high results) or which were not consistent with the clinical impressions of the referring physician. In addition, 6 samples were obtained from euthyroid women who had received Salpix for hysterosalpingograms. BEI determinations were also performed.

Based on the first fraction, the column technique gave results (Table 5) consistent with a euthyroid state in all 6 of the cases in which Salpix had

TABLE 5. COMPARISON OF BEI AND COLUMN METHODS APPLIED TO PATIENTS' SERA, WITH "ARTIFACTUAL" PBI VALUES*

Case	Serum level (μg. iodine/100 ml.)					Clinical findings	Remarks
	PBI method	BEI method	Column method (fraction)				
			1	2	3		
1	>25.0	19.0	4.6	2.7	12.6	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
2	>25.0†	16.3	5.5	4.3	9.7	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
3	>25.0	12.4	5.4	1.0	5.4	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
4	>25.0	11.1	4.6	1.8	1.2	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
5	23.0	4.8	5.2	1.0	1.2	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
6	21.0†	6.0	3.8	0.3	0.8	Euthyroid	Hysterosalpingography; Salpix 1 hr. prior.
8	>25.0	>25.0	6.3	2.1	0.0	Toxic adenomatous goiter	Itrumil for several weeks.
9	>25.0	17.0	7.1	2.0	0.9	Euthyroid	Telepaque or Orabilex shortly prior.
	22.0	4.7	4.6	0.2	0.4	Euthyroid	Hysterosalpingography (Ethiodol).
10	19.7†	9.5	>25.0	3.7	—	Euthyroid	
11	19.2‡	6.6	5.9	0.9	1.5	Treated hyperthyroidism	Receiving Lugol's solution.
12	19.0‡	3.5	3.3	0.3	0.3	Treated, iodide goiter	Asthma prep. containing KI until 7 days prior.
13	14.4	8.3	12.5	13.2	11.6	Hypothyroid or euthyroid	
14	13.2‡	6.3	5.6	0.8	0.0	Treated hypothyroidism	KI + thyroxine.
15	10.3‡	4.2	5.3	0.8	0.3	Euthyroid	

* PBI and BEI values are averages of duplicate determinations. Column values are from single determinations with duplicate iodine analyses on each fraction.
† Inorganic screening test values, 25–500 μg. per 100 ml.
‡ Inorganic screening test values, >500 μg. per 100 ml.

been administered one hour prior to obtaining the blood specimen. These same blood specimens all showed PBI values which were grossly elevated, and in only 2 instances (Cases 5 and 6) was the BEI value reliable. Both the column and BEI methods yielded values which appeared to be consistent with the clinical picture in Cases 11, 12, 14 and 15, in which the source of contamination was apparently inorganic iodine. Ethiodol for hysterosalpingography was the probable source of contamination in Case 9, and both tests gave clinically consistent results. In Cases 7 and 8, BEI results were falsely high, but fraction 1 of the column appeared to yield a clinically useful value. The BEI results in Cases 10 and 13 were in the physiologic range, but falsely high from a clinical viewpoint. In contrast, the column elution pattern was definitely abnormal and indicated failure of separation of thyroxine from the contaminant, thereby preventing a misleading result.

Another group of specimens (not shown in Table 5) analyzed by both methods consisted of sera in which both the PBI and inorganic iodine levels were in excess of 25 μg. per 100 ml. The source of contamination in these cases was not known. In 17 cases out of 18 studied, the BEI and column results were grossly elevated. No clinical data were available for evaluating the exception in which, with the column method, a physiologic but elevated value of 11.2 μg. per 100 ml. (with a normal elution pattern) was obtained, in association with a BEI value of 16.9 μg. per 100 ml.

DISCUSSION

The column method described in this paper for the determination of thyroxine in serum was found to be reliable in the presence of elevated inorganic iodine or iodotyrosine levels. In this respect it is equivalent to the butanol-extractable iodine method. Comparison of the column and BEI techniques with respect to their ability to determine thyroxine levels in the presence of *in vitro* additions of organic iodine compounds indicated some superiority of the column method, but neither was reliable in all cases. Only for additions of the compound Miokon was the BEI result superior to the column result. Since the foregoing studies were *in vitro* in nature (that is, involved additions of the compounds to serum) and because only one level of contamination was studied (1000 μg. per 100 ml. of serum, as iodine), the results with sera obtained from patients who received the compounds could be quite different from those obtained in this study, due to metabolite formation or different levels. The limited clinical correlation data obtained did confirm the ability of the column to determine thyroxine levels after hysterosalpingography utilizing the compound Salpix, and demonstrated the column method to be equivalent or better than the BEI method in other cases. Furthermore, one apparent advantage of the column technique over the BEI technique was the possibility of confirming the presence of

organic iodine contamination by virtue of an unusual elution pattern. In the normal pattern, over 90 per cent of eluted thyroxine should appear in the first of 3 successive 10-ml. fractions of the pH 1.4 eluate. There is no way of deciding whether or not a BEI result includes nonthyroxine iodine unless a grossly elevated value is obtained. In both the *in vitro* and the clinical correlation studies, falsely high BEI values within the physiologic range were obtained. These results could have proved misleading from a diagnostic point of view.

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THE FRACTIONATION OF EXOGENOUS AND ENDOGENOUS THYROID-STIMULATING HORMONE FROM HUMAN AND RAT PLASMA AND TISSUES*

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ABSTRACT

A method of fractionation is presented which permits the recovery of thyrotropic activity from serum, plasma and tissue homogenates even when present in concentrations as low as 0.002 mU per ml. A single application of the acid-salt fractionation method permits discard of about 75 per cent of the plasma proteins. After a second fractionation, as much as 95 per cent of the plasma proteins may be discarded without significant loss of thyrotropic activity. By this method, the potency of normal rat serum was found to be 0.02 mU per ml., and that of normal human plasma only 0.002 mU per ml. In hypothyroid rats and patients the levels were elevated, and in some patients with Graves' disease the levels were as high as 6000 times normal.

THREE recent reviews (1–3) have summarized attempts to measure the level of endogenous thyrotropin (TSH) in body fluids. Because of the limited sensitivity of available bio-assay methods and because of interference resulting from unidentified substances in plasma, some preliminary procedure for concentration of thyrotropin and its separation from other plasma proteins has usually proved necessary. In using our bovine thyroid-slice method for the assay of thyrotropin (4, 5) we found that nonspecific inhibition occurred when protein was present in a concentration greater than 2–5 mg. per ml. When a sample of plasma or serum was diluted enough to avoid this inhibition, the minimal detectable concentration of thyrotropin in the original sample was 0.2 U.S.P. mU per ml.[1] although the assay was sensitive to 0.007 mU per ml. (U.S.P. standard TSH in saline). Since

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[1] United States Pharmacopœia standard thyrotropin, milliunits per milliliter.

most body fluids contain less than 0.2 mU per ml. we searched for a method of concentrating thyrotropic activity with satisfactory recovery. A simple method was discovered which made use of the remarkable stability and solubility of thyrotropin in cold acid. For convenience, this method of fractionation is called the "acid-salt method."

MATERIALS AND METHODS

Materials

The bovine pituitary thyrotropin preparation used was either U.S.P. Thyrotropin Reference Standard[2] or, for injection, Armour Lot #6. In recovery experiments thyrotropin was added in quantities believed to approximate the concentrations that might be anticipated *in vivo*, that is, less than 1 mU per ml. Rat and human pituitary thyrotropin preparations were saline homogenates of fresh pituitary tissue, stored frozen.

A fresh pool of human plasma was obtained in a single morning from 15 normal volunteers who manifested no evidence of thyroid or other disease. Immediately after collection, the plasma was cooled, mixed, divided into convenient aliquots, frozen, and stored.

Tissue homogenates were prepared at 4° C. with a conical tissue grinder,[3] using isotonic Krebs-Ringer phosphate buffer with $10^{-3}M$ propylthiouracil (hereafter abbreviated "KRPP"). Aliquots were then frozen.

Serum and tissue were obtained from normal and hypothyroid Sprague-Dawley male rats each weighing about 250 Gm. The rats were made hypothyroid by giving them either a low-iodine diet for two months, or 0.05 per cent propylthiouracil in the drinking water for two or more weeks.

Method of fractionation

The sample of plasma, serum or tissue homogenate was thawed at about 22° C. and then kept at 0–4° C. during all subsequent steps. If the initial potency was sufficiently high, a diluted sample (*i.e.*, less than 4 mg. of protein per ml.) was assayed concurrently with aliquots fractionated by the acid-salt method.

The acid-salt method consisted of diluting the serum or plasma with an equal volume of water and then adding cold 2 *M* hydrochloric acid drop by drop with constant stirring, to bring the pH to 1.2. After twenty minutes, a predetermined amount of sodium chloride was added, with stirring, to yield a final concentration of 1 *M*. The mixture was then sedimented in a refrigerated angle-head centrifuge at 4000 rpm for forty-five minutes. The supernatant was poured off and saved; the precipitate was washed three times with minimal volumes of cold 1 *M* sodium chloride solution (pH 1.2) and discarded. The washes were pooled with the first supernatant, and cold 2 *M* hydrochloric acid was added until a pH of 0.5 was reached. Sodium chloride, in half the amount used for the first precipitation, was then added and the solution was allowed to stand overnight at 4° C. The precipitate was sedimented by spinning in an angle-head centrifuge at 4000 rpm for two hours. The precipitate was washed with *M* sodium chloride at pH 0.5 and discarded. The supernatants were combined and dialyzed for eighteen hours at 4° C. against four changes of KRPP. Dialysis was used to neutralize the supernatant in order to keep the volume of the sample at a minimum and because it was found that adding

[2] Obtained from U.S.P. Reference Standards, New York 16, New York.

[3] Kontes Glass Co., Vineland, N. J.

concentrated alkali destroyed the thyrotropic activity even when cold alkali was added drop by drop with careful stirring and refrigeration. If lyophilization or flash evaporation[4] was planned, the final two changes of dialysis were against KRPP diluted with as much as 10 volumes of water in order to reduce the salt content. The dilution of the KRPP was adjusted so that the final concentration would be isotonic for assay. Lyophilized samples were prepared for assay by dissolving in distilled water and dialyzing for eighteen hours against KRPP to assure the isotonicity and neutrality required for assay. It was not considered necessary to evaluate systematically each factor in this procedure except to demonstrate that recovery was not impaired.

The nitrogen content of aliquots of the initial material and the final supernatant was measured by the micro-Kjeldahl method. These nitrogen analyses revealed that 60–85 per cent (usually 75 per cent) of the protein was removed by acid-salt fractionation.

In several attempts to obtain even greater concentration, plasma samples were subjected to acid-salt fractionation and then to trichloracetic acid precipitation, using a final concentration of 5 per cent. This eliminated at least 99 per cent of the original plasma protein, but variable loss of thyrotropic activity was noted and sometimes the product contained inhibitors which interfered with the assay response. An alternative method of further concentration was successfully achieved by making a concentrated solution of the lyophilized or flash-evaporated sample and repeating the complete acid-salt fractionation. This second fractionation eliminated 75–80 per cent of the remaining protein. Thus, in combination, the two steps removed 95 per cent of the initial protein.

Bio-assay method

Thyrotropic activity was bio-assayed by the weight-response of surviving bovine thyroid slices (5). When $10^{-3}M$ propylthiouracil was present in the incubation medium, this assay usually measured concentrations as low as 0.007 mU per ml. of incubation medium. The simplicity of the assay enables one to use 200 beakers, each containing an individual bovine thyroid slice, all obtained from a single gland. Because of variations in the response from day to day, a five-dose standard curve was run simultaneously with the unknown samples in quintuplicate each day. The unknowns were run at several dose levels, spaced at three- or fourfold intervals. This permitted a comparison of the slopes of the responses of the standard and unknown. The 95 per cent ($P=0.05$) confidence limits of the estimate of potency were calculated according to the method of Bliss (see ref. 5). These confidence limits are included in parentheses after the mean potency values. An aliquot of the same thyrotropin preparation used in a recovery experiment was used as the standard in the assay of the unknowns.

RESULTS

Recovery of exogenous thyrotropin

Table 1 summarizes the results of a series of experiments in which rat, human or beef thyrotropin was added to rat or human plasma in concentrations varying from 0.05 to 11.0 mU per ml. In each experiment all the added TSH was recovered within the limits of assay accuracy. As shown in Figures 1, 2 and 3, the assay lines for the recovered activity were always parallel to those for the standard. Figure 1 illustrates the recovery of TSH added

[4] The temperature of the water bath in which the flash evaporator flask was immersed was controlled so that the temperature of the sample never exceeded 10°C.

TABLE 1. ACID-SALT FRACTIONATION OF TSH

Sample	Vol. (ml.)	Endogeneous TSH level (mU/ml.)	Added TSH* (mU/ml.)	Fraction**	Potency (mU/ml.)	Recovery (%)
Rat serum	10	0.02	10	S	12.0 (6.8–21.0) †	109 (61–188)
				P	0.12	1
Human serum	10	<0.01	2	S	1.9 (0.86–4.0)	97 (43–199)
				P	0.029 (0.008–0.07)	1
Human plasma	930	<0.01	0.054	S	0.095 (0.072–0.13)	176 (133–241)
Human plasma	50	<0.01	0.2	S	0.17 (0.12–0.24)	85 (60–120)
				P	0.008 (0.004–0.012)	4
Human plasma	50	<0.01	0.33 (h)	S	0.23 (0.16–0.33)	70 (50–100)
				P	0.033 (0.016–0.045)	10
Human plasma	114	<0.01	0.05	S‡	0.043 (0.032–0.056)	85 (63–112)
Rat pituitary		14 (11–18) §	0	S	9.1 (6.9–12)	—‖
Human pituitary		0.76 (0.56–1.1) §	0	S	0.57 (0.41–0.83)	—‖
Rat kidney¶		0.026 (0.015–0.053) §	0	S	0.041 (0.028–0.06)	—‖

* Bovine TSH, Armour Lot #6 or U.S.P. reference standard, except when human TSH was used as marked (h).
** S = Supernatant; P = Precipitate.
† 95% confidence limits in parentheses.
‡ Sample fractionated twice (see text).
§ In terms of mU/mg. wet weight.
‖ No significant difference between fractionated and unfractionated samples.
¶ Kidney removed 15 minutes after injection of TSH (see text.)

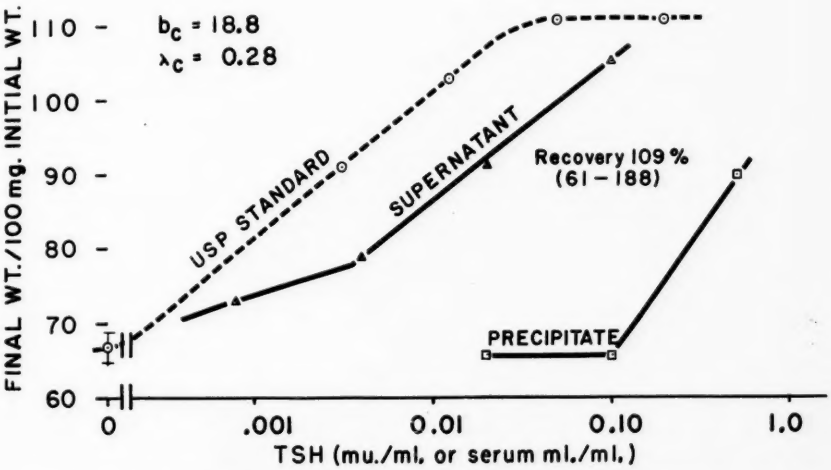

FIG. 1. Recovery of TSH added to rat serum to make a concentration of 11 mU/ml. The supernatant contained 109 per cent (61–188 per cent) of the added activity. The precipitate contained less than 1 per cent.

In all figures each point is the mean of 5 replicate thyroid slices. The standard error of the mean is illustrated for the control group only, being similar for each of the other points. The combined slope (b_c) and combined index of precision (λ_c) are given for each assay. The values for fractionated activity are parallel to those for the standard; this was verified statistically in each assay.

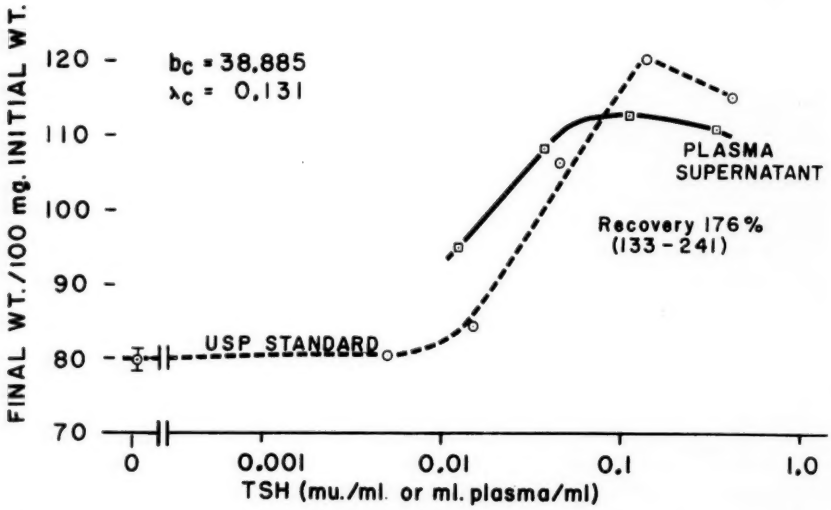

FIG. 2. The recovery of TSH added to human plasma to make a concentration of 0.054 mU/ml. The final supernatant was lyophilized in order to concentrate the sample, which was found to contain 176 per cent (133–241 per cent) of the initial activity.

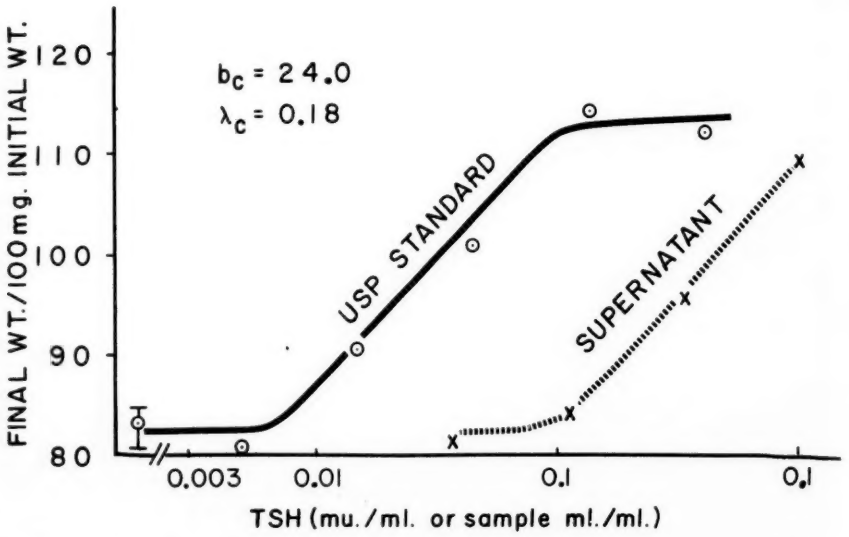

FIG. 3. The recovery of TSH added to human plasma to make a concentration of 0.05 mU/ml. The supernatant was fractionated twice, using flash evaporation to concentrate the first supernatant (see text). The final sample contained 85 per cent (63–112 per cent) of the initial activity.

to normal rat serum[5] to make a concentration of 11 mU per ml. The supernatant contained 109 per cent of the added TSH (95 per cent confidence limits; 61–188 per cent). The precipitate contained less than 1 per cent. Figure 2 shows the measurement of a much lower concentration (0.05 mU per ml.) of TSH in human plasma. In this experiment, a 54-mU portion was added to 930 ml. of normal fresh pooled plasma. Assay revealed 0.095 mU per ml. (0.072–0.13 mU), suggesting the possible measurement of some endogenous thyrotropin. Another sample of normal human plasma containing 0.05 mU of TSH per ml. was subjected to acid-salt fractionation. The supernatant was neutralized, concentrated and subjected to a second acid-salt fractionation. More than 94 per cent of the protein was discarded. Figure 3 shows that 85 per cent (63–112 per cent) of the thyrotropin was recovered. No significant endogenous activity was detected.

Fractionation of endogenous thyrotropin

Thyrotropic activity was satisfactorily recovered from tissue homogenates by acid-salt fractionation. Table 1 shows the assay of samples of human pituitary, rat pituitary, and homogenates prepared from rat kidneys removed fifteen minutes after the intravenous injection of bovine thyrotropin.[6] Because the potency of these samples was high enough to permit direct assay, the results with the acid-salt method were compared with the assay of the untreated sample as shown in Table 1. In each experiment the recoveries were satisfactory.

Blood from rats made hypothyroid by a low-iodine diet or by propylthiouracil contained TSH concentrations of 0.9 to 4.0 mU per ml. of serum, depending upon the duration and severity of the hypothyroidism. One example is shown in Figure 4. The endogenous activity assay line was parallel with that of the U.S.P. standard. The supernatant contained 99 per cent of the recovered activity and had a potency of $0.93(0.60-1.45)$ mU per ml.

To demonstrate that the acid-salt fractionation caused no loss of endogenous activity, a pooled sample of serum from hypothyroid rats was divided, half being subjected to acid-salt fractionation and the other half dialyzed and assayed directly. This was possible because of the sample's high potency (0.68 mU per ml.). Figure 5 shows that the acid-salt method recovered the full potency; the fractionated sample had a potency 118 per cent (66–142 per cent) of the directly assayed sample.

In several determinations of pooled normal rat serum the average potency was 0.021 mU per ml., with a range of 0.010 to 0.044 mU. Samples of

[5] Later assay showed that this pool of normal rat serum contained 0.02 mU per ml. of endogenous thyrotropic activity.

[6] Armour Lot 6.

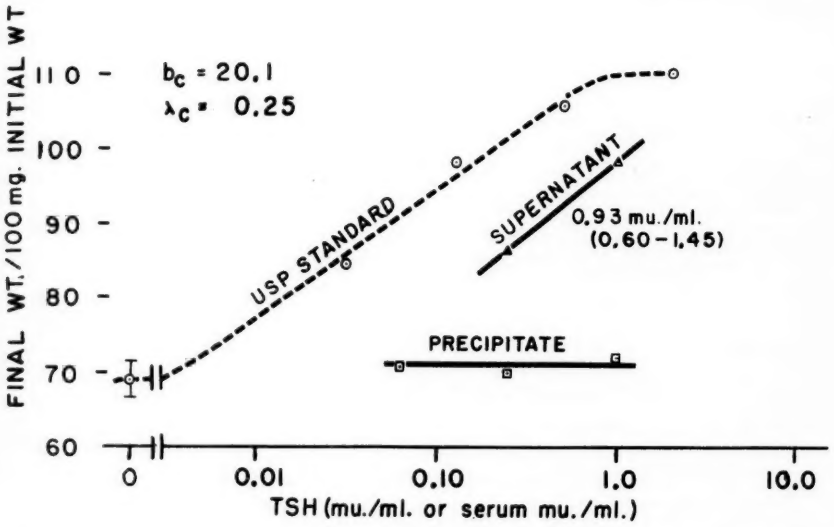

FIG. 4. The recovery of endogenous TSH (0.93 mU/ml.) from the serum of hypothyroid rats. The supernatant contained all the activity; none was detected in the precipitate.

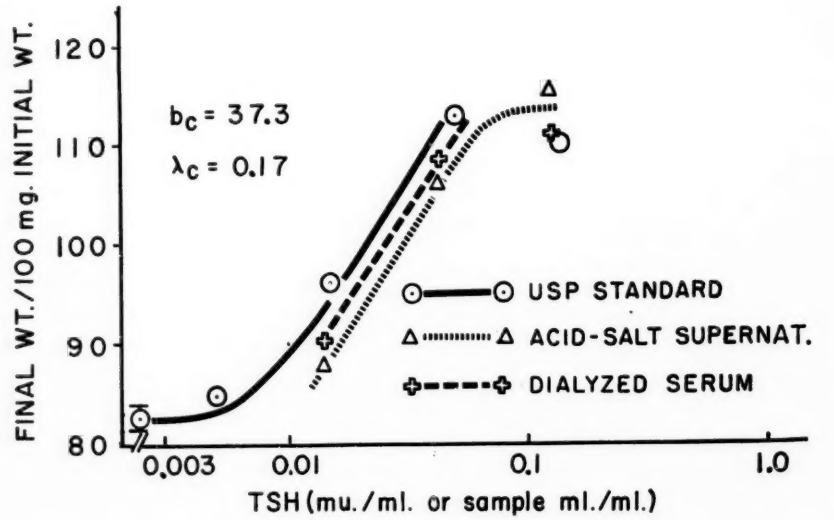

FIG. 5. The recovery of endogenous TSH (0.68 mU/ml.) from the serum of hypothyroid rats (not the same as used for Fig. 4), before and after fractionation. The potencies as assayed by acid-salt fractionation and the direct method did not differ significantly (see text).

serum (60 ml. to 120 ml.) were obtained from the blood of 5 patients with untreated hypothyroidism. Assay of each sample showed that the TSH concentration varied from 0.01 to 0.07 mU per ml., with an average of 0.04 mU. The serum of patients with untreated and treated Graves' disease, including a sample rich in the "abnormal thyroid stimulator,"[7] sometimes had a much higher potency (up to 7 mU per ml.). These studies will be reported elsewhere.

After several failures to detect any TSH in the plasma of normal men, a 1000-ml. pool of fresh plasma was reduced to a water-clear 50-ml. sample by double acid-salt fractionation. This sample contained only 2 mU of thyrotropic activity, indicating an original concentration of 0.002 mU per ml. Repetition of this experiment yielded the same result.

DISCUSSION

The fractionation method described here affords complete recovery of added human and bovine pituitary thyrotropin from plasma or tissue homogenates. In addition it permits some separation from other proteins and from substances which interfere with the assay response. The fractionation is based upon the remarkable stability and solubility of thyrotropin in strong acid and salt solutions, including solubility in trichloracetic acid, as recently reported by Pierce *et al.* (6).

The thyrotropic activity measured in hypothyroid rat serum was similar to the amount reported by D'Angelo (7); however, our measurement in normal rat serum gave much lower values than he obtained. This difference may be accounted for by differences in strain, sex, and our high (25°–27° C.) animal-room temperature which would be expected to result in lower values.

The results obtained with acid-salt fractionation lead to the conclusion that the TSH potency of normal human plasma is about 0.002 mU per ml. Because pure thyrotropin[8] was found to have an activity of 77 (55–107) mU per μg. by our assay, we concluded that probably there is less than 40 millimicrograms of thyrotropin in a liter of normal plasma; or, based upon a molecular weight of 27,000 (6), about a 10^{-12} molar concentration. If this value is correct, the many past failures (2) to detect activity are understandable.

However, it is important to note the disparity between this estimate of the normal level and that reported by Di George *et al.* (8), Gilliland and Strudwick (9), Bottari (10), Bates *et al.* (11) and McKenzie (12), who have reported levels in the range of 0.25 to 0.50 mU per ml. The first 3 methods cannot detect activity below this level; therefore their assay estimates are

[7] As assayed by S. Werner, J. M. McKenzie and H. D. Purves.

[8] Generously supplied by Dr. J. G. Pierce (6).

based upon a single point and cannot be subjected to the usual test for parallelism. Several laboratories in this country, using McKenzie's or Bottari's assay methods, have failed to detect any thyrotropic activity in normal human serum. Bates has recently (13) revised his estimate of the level in human serum downward fiftyfold, to 0.01 mU per ml. Purves and Adams (14) also now report 0.012 mU per ml. in a pool of normal serum. These levels are probably not significantly different from ours. As has been the case with other hormone bio-assays, it may be that with each increase in assay sensitivity the estimate of the normal blood content will be revised downward.

It is also possible, however, that the bovine thyroid-slice assay may be qualitatively unlike other assay methods. It may be measuring a different aspect of the spectrum of thyrotropic effects which may not always be present in a given sample to the same degree.

There is indirect evidence to support our low estimate. Greer and Shull (15) and later, Einhorn and Larsson (16) demonstrated that in normal subjects the smallest dose of exogenous thyrotropin that causes a response is approximately 200 to 600 mU per day—suggesting that this dose is slightly greater than the normal pituitary secretion rate. When this value is combined with estimates of the disappearance rate of thyrotropin in the plasma of normal subjects as reported by Bakke *et al.* (17), one may calculate the circulating plasma level in a steady state. If the disappearance rate has a half-time of thirty-five minutes and is distributed in a plasma volume of 2.67 liters, approximately 2 per cent of this pool will turn over each minute. Using the foregoing estimate of a pituitary secretion rate of 200 to 600 mU per day (or 8.3 to 25 mU per hour), the plasma level in a steady state would be 0.003 to 0.008 mU per ml.—not significantly different from our assay value.

Although the acid-salt method seems to be suitable for application to rat serum and tissues and for detecting elevated levels in human plasma, it is of no use in measuring either normal or subnormal levels in a single sample of human plasma. Application of the method to analysis of samples from patients with various clinical disorders is in progress.

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6β-HYDROXYCORTISOL AND OTHER POLAR CORTICOSTEROIDS: MEASUREMENT AND SIGNIFICANCE IN HUMAN URINE*†

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ABSTRACT

A detailed description of extraction and paper chromatographic techniques for the measurement of urinary 6β-hydroxycortisol is presented, together with experimental data from which this and other separation procedures have been devised. 6β-Hydroxycortisol appears to be the chief unconjugated urinary metabolite of cortisol. Increased levels, both absolutely and in relation to total steroid excretion, were noted in cases of Cushing's syndrome and after administration of ACTH. Evidence for the presence of several new urinary steroids is presented, and definitive identification of one, 6α-hydroxycortisol, is reported in human urine for the first time.

THE presence of highly polar reducing corticosteroids in chromatograms of urinary extracts has been noted for several years. However, methods conventionally used for corticoid analysis do not extract or separate these compounds adequately and accurate appraisal of their role in adrenal steroid metabolism has been difficult. Burstein, Dorfman and Nadel, in 1954, were the first definitely to identify a $C_{21}O_6$ steroid—6β-hydroxycortisol (6β,11β,17α,21-tetrahydroxy-Δ^4-pregnene-3,20-dione)—in guinea-pig urine (1), and later in normal human urine (2, 3). Touchstone, Kasparow and Rosenthal (4) tentatively identified 6β-hydroxycortisol as one of the products of human adrenals incubated *in vitro* in autologous

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plasma. More recently, Ulstrom and co-workers (5, 6) reported that 6β-hydroxycortisol is an important metabolite of cortisol in the newborn human, and also adduced evidence for the presence of 2α-hydroxycortisol (2α,11β,17α,21 tetrahydroxy-Δ^4 pregnene-3,20-dione) and possibly also the reduced form of 6β-hydroxycortisol (3α,6β,11β,17α,21-pentahydroxy-Δ^4 pregnene-20-one) in the urine of newborn infants.

In a previous study (7), based upon the development of quantitative methods, normal adult excretion levels of 6β-hydroxycortisol were reported, and this compound was found to be the most abundant unconjugated steroid in human urine. Excretion was noted to be elevated in the third trimester of normal pregnancy, and greatly increased in the toxemia of pregnancy. The present communication 1) describes techniques for the measurement of 6β-hydroxycortisol and for the general study of polar corticosteroids, 2) reports identification of at least one new steroid in human urine, and 3) presents data obtained in cases of Cushing's syndrome.

MATERIALS AND METHODS

Collection and storage of urine

The 24-hour collections were adjusted to approximately pH 4.5 with glacial acetic acid and stored at 4° C. if not used fresh. Periods of storage in general did not exceed a few weeks at most. Specimens tested at intervals during storage showed no appreciable changes.

Equipment

Solvents were redistilled before use.

Chromatography paper. Sheets of Whatman #3MM or #2 paper were cut into strips 15 cm. wide, and washed by descending chromatography for two to three days with a methanol:water (1:1) mixture, followed by pure methanol for one day.

Extraction and washing procedures

Volumes of urine representing 10 per cent of the 24-hour output were usually chosen for analysis. Twenty per cent by weight of sodium sulfate was dissolved, by stirring, in the urine sample; brief warming to not more than 35° C. was occasionally necessary to assist solution. The mixture was then subjected to extraction two times with twice its volume of ethyl acetate (4:1 ratios of ethyl acetate to urine were sometimes used to reduce emulsion formation). The combined ethyl acetate extracts were washed twice with 1/20 volume of a solution of 1.0 *N* sodium hydroxide to which had been added 15 per cent by weight of sodium sulfate, once with 1/20 volume of a 0.5 per cent acetic acid solution containing 15 per cent by weight of sodium sulfate, and once with 1/100 volume of distilled water. All washings were carried out in a separatory funnel under an atmosphere of nitrogen. The ethyl acetate extract was taken to dryness in a flash evaporator at 37° C. and the residue transferred to a test tube with two successive 5–10 ml. portions of an ethyl acetate:methanol mixture (1:1) followed by 5–10 ml. of pure methanol. The extract was partly evaporated at 37° C. under a stream of filtered air, and finally dried under nitrogen.

In preparation for chromatography, the dried residue was dissolved in exactly 5 ml. of ethyl acetate:methanol (1:1). A 3.5–ml. portion of this solution, representing 7 per cent of the 24-hour output, was transferred to another test tube; the contents of this and the original test tube, now containing 3 per cent of the 24-hour total, were dried under nitrogen.

Paper chromatography

Two new highly polar solvent systems, developed for the separation of $C_{21}O_6$ and similar steroids, were used in the present study:

System Y: Ethyl acetate:chloroform:methanol:water (25:75:50:50), and

System Z: Benzene:tert.-butyl alcohol:water (70:43:86).

The solvents are shaken together at room temperature and allowed to separate. The mobile phase is the bottom layer in the Y system, and the top layer in the Z system. The use of tert.-butyl alcohol as a solvent was suggested by the work of Eberlein and Bongiovanni (8). Descending chromatography was carried out at room temperature according to the general method of Bush (9). Circular glass tanks were used, lined with Whatman #3MM filter paper dipping into a layer of stationary phase at the bottom. A beaker or trough containing some mobile phase was placed in the bottom of the tank, and one or more wads of paper saturated with mobile phase were hung near the top.

With the aid of a few drops of methanol:methylene chloride (2:1) mixture, the extract representing 7 per cent of the 24-hour urine was streaked onto a limb (3.8 cm. wide) of Whatman #3MM chromatography paper, and the 3 per cent portion was streaked onto an adjacent 1.3-cm. limb to serve as a marker. Next to these was a 2.5-cm. limb carrying 6β-hydroxycortisol and other reference standards. The dry paper was equilibrated for two to three hours in System Y. Then a 40-cm. run, requiring approximately three hours, was carried out. After drying for a few minutes, the paper was scanned briefly under a 254 mμ light to locate the ultraviolet-absorbing bands. The 3 per cent strip was divided longitudinally; half was dipped through 2.5 *N* sodium hydroxide, dried in an oven at 60° C. for an hour, and examined for fluorescence under a 365 mμ light; the other half was treated with alkaline blue tetrazolium (BT). A parallel area on the 7 per cent strip, corresponding to the location of the 6β-hydroxycortisol and to a BT-positive, soda-fluorescent area on the 3 per cent strip, was cut out and eluted with methanol:water (9:1) as previously described (10).

The eluate from the 7 per cent strip was dried, dissolved in ethyl acetate:methanol (1:1), and divided into portions usually representing 3 per cent or 4 per cent of the 24-hour output. These two portions were dried and applied respectively to 1.2-cm. and 2.5-cm. limbs of Whatman #2 paper, adjacent to a limb carrying standards. Equilibration was carried out overnight in system Z, followed by a 40-cm. run lasting four to five hours. The 6β-hydroxycortisol band was spotted on the standard and on the 3 per cent marker limbs as described, and a corresponding area of the 4 per cent limb was cut out and eluted.

Quantitation

Two equal dried portions of the final eluate, usually representing 1 per cent each of the 24-hour urine, were dissolved in separate 0.3-ml. aliquots of freshly prepared 11.2 *M* sulfuric acid:ethanol mixture (2:1), one of which contained phenylhydrazine dissolved in the sulfuric acid before mixing with ethanol (32.5 mg. of phenylhydrazine per 50 ml. of 11.2 *M* sulfuric acid). Readings at three wavelengths, subtraction of blank readings, and quantitation were carried out as previously described (10) in comparison with a 6β-hydroxycortisol standard.

Total 17-ketogenic steroids were determined on separate samples of urine by a modification of the borohydride method of Appleby *et al.* (11) similar to that described by Sobel *et al.* (12).

Preparation of steroid derivatives

Steroid acetates were formed by adding approximately 20 μl. of acetic anhydride and 40 μl. of redistilled pyridine to each 5 μg. of steroid. The tubes were tightly stoppered and were allowed to stand in the dark for fifteen hours. The reaction was stopped by adding an excess of methanol:benzene (1:1).

Steroids were oxidized with sodium bismuthate and chromic acid by the methods of Bush and Willoughby (13).

EXPERIMENTAL

Extraction of polar steroids from urine

In order to establish quantitative data for urinary levels of corticosteroids more polar than the $C_{21}O_5$ compounds, it was necessary to determine the completeness of extraction from aqueous solutions. Burstein (14) had earlier shown the unsuitability of chloroform and methylene chloride for extracting polar corticoids, and suggested the use of ethyl acetate. However, 6β-hydroxycortisol is partitioned equally between ethyl acetate and water, and the use of repeated extractions or of large volumes of solvent is therefore necessary for satisfactory extraction of the steroid. After considerable investigation it was decided to retain this solvent, which has many desirable properties, but to increase its efficiency by a salting-out process. As can be seen from the first three partition coefficients in Table 1, the addition of 20 per cent sodium sulfate to the aqueous phase greatly altered the distribution of 6β-hydroxycortisol, so that over 90 per cent was removed by a single extraction with an equal volume of ethyl acetate.

TABLE 1. STEROID PARTITION COEFFICIENTS

$$K = \frac{\text{Steroid conc. in organic phase*}}{\text{Steroid conc. in aqueous phase}}$$

Solvents	Steroids		
	6β-OH-cortisol	6β-OH-cortisone	Cortisol
Ethyl acetate/water+20% Na_2SO_4	11.5	24	
Ethyl acetate/water+10% Na_2SO_4	4.1	13.3	
Ethyl acetate/water	1.13	2.0	12.2†
50% Ethyl acetate+50% *n*-heptane/water	0.095		1.4
33% Ethyl acetate+66% *n*-heptane/water	0.046		0.44
25% Ethyl acetate+75% *n*-heptane/water		0.18	0.16

* Determinations performed at room temperature on solvents not previously saturated with one another.

† Value reported by Burstein (14).

In the final procedure, favorable partition ratios were maintained during the washing procedures by the addition of sodium sulfate to the aqueous alkali and acid solutions. Inspection of chromatograms revealed that many urinary compounds still more polar than 6β-hydroxycortisol, appearing in considerable quantities with the method as described, are undetectable when the salting-out procedures are omitted.

Recovery experiments

A multi-step procedure such as this involves certain unavoidable losses at every stage. Over-all recovery figures, for which final values were corrected, were checked frequently throughout these studies by means of known amounts of 6β-hydroxycortisol dissolved in 100–200 ml. of distilled water and carried through the same procedure as the urines. Recovery experiments were also carried out, in which 6β-hydroxycortisol was added to urine. Consistent recoveries averaging 60 per cent were obtained in a series of determinations performed simultaneously with the routine urine assays, and this correction figure was generally applied to the final results. In one phase of the work, recoveries aa low as 50 per cent were noted, apparently owing to minor differences in technique. A study of each stage in the method (extraction, washing, chromatography and elution) revealed that the greatest losses were incurred during the alkali washes. Reduction in strength of the alkali improved the recovery somewhat, but resulted in more voluminous and unwieldy extracts. An attempt to substitute sodium carbonate for sodium hydroxide produced extracts too heavy and impure for satisfactory chromatography. Recovery values for the elution step, which were improved by the substitution of methanol:water (9:1) for pure methanol, exceeded 90 per cent. Duplicate determinations carried out on separate aliquots of the same urine never varied more than ±10 per cent.

Procedures for isolation of steroids from larger urine volumes

Additional procedures were devised to remove much of the contaminating material from large residues prior to chromatography, and to permit the crystallization of polar steroids from 24-hour or larger volumes of urine (Fig. 1). Amounts ranging up to several liters of urine were evaporated to one-quarter or less of their original volume in shallow pans at 30°C under a stream of air. Sodium sulfate was then added, and the ethyl acetate extractions and alkali washes performed as described. The final ethyl acetate solution, which at this point was sometimes reduced in volume by partial evaporation under vacuum, was mixed with twice its volume of *n*-heptane. The resulting solution was extracted three times with one-third volume of distilled water. Large amounts of contaminating nonpolar material are left behind in the organic phase. However, as shown by the partition coeffi-

PURIFICATION OF RESIDUES FROM LARGER URINE VOLUMES

Urine concentrated by evaporation
↓
10–20% Na_2SO_4 dissolved in urine
↓
Extracted with ethyl acetate
↓
Alkali and other washes
↓
Ethyl acetate solution mixed with 2 volumes *n*-heptane
↓
Mixtures extracted 3 times with ⅓ volume water
↓
20% w/v Na_2SO_4 dissolved in combined water extracts
↓
Mixture re-extracted twice with equal volumes ethyl acetate
↓
Taken to dryness

FIGURE 1

cients in Table 1, 6β-hydroxycortisol passes almost quantitatively into the aqueous phase. The combined water extracts were then treated with sodium sulfate, re-extracted with ethyl acetate, and taken to dryness. If nonpolar lipid material was present in very high concentrations, as with plasma extracts, the final ethyl acetate solution was mixed again with heptane and the extractions repeated.

This general process, of solute transfers between two phases by means of alternate and opposite displacement of the distribution ratios, is applicable to the purification of a wide variety of polar substances. It can achieve a marked reduction in the weight of the extracts, with low losses and relatively few and simple manipulations. By appropriate choice of sodium sulfate and heptane concentrations, based on partition coefficient determinations, or the use of certain solvents other than ethyl acetate, the procedure may be made more or less highly selective for a given substance or polarity spectrum. As with all extraction processes, various substances present in biologic extracts may alter to some extent the partition coefficients obtained with the use of pure solutions; such values also become inapplicable when solutions approach saturation.

Extracts prepared by the foregoing method were streaked across the full width of one or more 15×58-cm. washed sheets of Schleicher & Schuell #2071 chromatography paper[1], and chromatographed in systems Y and Z. Equilibration and running times were somewhat longer than for thinner papers, and over-runs of up to thirty-eight hours were used to achieve separation of steroids not otherwise easily resolved. Elution was performed by repeated washing in methanol:water (9:1) of areas which had been cut into small pieces. Choice of this unusual paper was made in an effort to find

[1] Schleicher & Schuell, Keene, New Hampshire.

a supporting medium which would combine very high loading capacity with good resolution. With its aid, crystallization of some urinary steroids was accomplished after as few as two chromatographic runs, followed by a brief transfer process of the type described, to remove paper contaminants.

RESULTS

Isolation and identification of steroids

Many highly polar substances which produce color reactions characteristic of steroids were observed routinely on chromatograms of urinary extracts prepared by the techniques described. Identification of some of them is discussed here.

1. *6β-Hydroxycortisol.* This compound was noted on chromatograms of all normal urine specimens studied to date. In every case, by the methods of detection used, it was the most abundant free steroid present. Identity was based in all cases on Rf values in two chromatographic systems, plus the presence of ultraviolet absorption, soda fluorescence, and positive reactions to blue tetrazolium and Porter-Silber reagents. Numerous samples were converted to the diacetate and to the bismuthate and chromic-acid oxidation derivatives, all of which showed chromatographic mobilities and color reactions identical with their authentic counterparts. Absorption spectra in sulfuric acid (15) of several specimens were the same as those of the reference standard. From two urine collections—those of a young man who ingested relatively large amounts of cortisol and a 45-year-old man with functioning adrenal carcinoma—substances were isolated which, after crystallization from acetone-methanol, each yielded an infrared spectrum (performed through the courtesy of Dr. Samuel Solomon and Dr. Seymour Lieberman) identical with that of authentic 6β-hydroxycortisol.

6β-Hydroxycortisol, in very small quantities, was tentatively identified in the peripheral blood of a normal adult male following two days of stimulation with 40 units of ACTH every twelve hours. A 560-ml. sample of whole blood, processed by techniques similar to those described, yielded a faint but sharply defined band having the exact chromatographic mobilities of 6β-hydroxycortisol in two systems, and exhibiting the soda fluorescence and blue tetrazolium reactions. Quantities were not sufficient to permit further characterization.

2. *6α-Hydroxycortisol.* Apparent widening of the urinary 6β-hydroxycortisol band in both the Y and Z systems, noticeable when larger amounts had been chromatographed, suggested that another closely moving steroid might be present. Further investigation led to the isolation of the 6α-isomer. The presence of this steroid in guinea-pig urine had been suggested by Burstein and Dorfman (16) and it has been recently tentatively

identified in human urine (7). As can be seen from the Rf values in Table 2, the chromatographic sequence of the two isomers is reversed in the Y and Z systems, a fact which assists in their isolation. Their Rf values, although similar, are still sufficiently different to permit complete separation after repeated chromatography.

The diacetates of 6α- and 6β-hydroxycortisol tend to separate less easily chromatographically than do the parent compounds. On the other hand,

TABLE 2. RUNNING RATES OF STEROIDS IN PAPER CHROMATOGRAPHIC SYSTEMS

$$R_f = \frac{\text{Distance of steroid from origin*}}{\text{Distance of solvent front from origin}}$$

Steroid	System†			
	Y	Z	**	B_5
6α-Hydroxycortisol	.39	.53		Origin
6β-Hydroxycortisol	.43	.49	.06	Origin
6β-Hydroxycortisone	.69	.75	.21	.03
20α-Hydroxycortisol (11β,17α,20α,21-tetrahydroxy-Δ4-pregnene-3-one)	.66	.74	.29	.06
20β-Hydroxycortisol (11β,17α,20β,21-tetrahydroxy-Δ4-pregnene-3-one)	.66	.77	.35	.07
2α-Hydroxycortisone	.74	.78	.42	
THF (3α,11β,17α,21-tetrahydroxy-pregnane-20-one)	.83	.82	.38	.14
THE (3α,17α,21-trihydroxy-pregnane-3,20-dione)	.88	.85	.59	.23
6β-Hydroxy-11-desoxycortisol (6β,17α,21-trihydroxy-Δ4 pregnene-3,20-dione)			.49	.18
Cortisol	.88	.85	.64	.25
Cortisone	.92	.90	.81	.44
6α,11β-Dihydroxy-Δ4-androstene-3,17-dione				.16
6β,11β-Dihydroxy-Δ4-androstene-3,17-dione				.25
6β-Hydroxy-Δ4-androstene-3,11,17-trione				.60
6α-Hydroxycortisol diacetate (6α,21-diacetoxy-11β,17α-dihydroxy-Δ4-pregnene-3,20-dione)				
6β-Hydroxycortisol diacetate (6β,21-diacetoxy-11β,17α-dihydroxy-Δ4-pregnene-3,20-dione)				

* All steroids run on Whatman No. 3MM paper for 40 ± 2 cm. at room temperatures. Slightly different values obtained on Whatman No. 2 paper.

† Y = Ethyl acetate:chloroform:methanol:water [25:75:50:50].
Z = Tert.-butyl alcohol:benzene:water [43:70:86].
** = Chloroform:benzene:methanol:water [50:50:50:50] (17).
B_5 = Benzene:methanol:water [100:50:50] (9).

17-ketosteroid derivatives (6α- and 6β-,11β-dihydroxy-Δ4-androstene-3,17-dione) prepared by bismuthate oxidation have rather widely differing mobilities in the Bush B_5 system (9). The formation of these derivatives offers one of the best means of differentiating 6α- from 6β-hydroxycortisol. Chromic-acid oxidation is of no help in this respect, yielding a 6-keto derivative which is the same for both isomers.

6α-Hydroxycortisol was noted in all normal and abnormal urine specimens which were specifically examined for its presence. This ultraviolet-

absorbing steroid was first located by its identical chromatographic mobility in the Y and Z systems as a synthetic standard of the free alcohol. It exhibited the blue tetrazolium and soda fluorescence reactions. The 17-ketosteroid derivative prepared by sodium bismuthate oxidation had a chromatographic mobility, in two systems, identical with that of a similarly treated standard. 6α-Hydroxycortisol isolated from the urines of 2 normal men after cortisol administration and from the urine of a patient with adrenal carcinoma, was further characterized by the chromatographic behavior of the acetylated derivatives. The sample from the carcinoma patient produced a spectrum in concentrated sulfuric acid, identical with that of a synthetic standard. The free steroid from the 3 subjects was pooled and crystallized from acetone-methanol. An infrared spectrum (determined through the courtesy of Dr. Thomas F. Gallagher, Dr. David K. Fukushima and Mrs. Beatrice S. Gallagher) was described as follows: "This spectrum . . . had all the bands present in the standard spectrum of 6α,11β,17α,21-tetrahydroxy-Δ^4-pregnene-3,20-dione and no other absorptions." This steroid has not, to the authors' knowledge, been previously identified in human urine.

When 6β-hydroxycortisol is purified and measured according to the routine method, it will be contaminated with small amounts of 6α-hydroxycortisol. Though precise quantitation of the latter steroid has not been attempted, estimates by a variety of methods indicate that it does not usually represent more than 5–10 per cent of the total 6-hydroxycortisol.

3. *Other steroids.* Less definitive characterization was obtained of other polar corticosteroids not previously known to be constituents of human urine. From the urine of 2 patients with adrenal carcinoma, a soda-fluorescent, blue tetrazolium-reducing compound was isolated which had mobilities in 3 systems (Y, Z, and Bush's (17) chloroform:benzene:methanol:water, 1:1:1:1, system), identical with that of authentic 6β-hydroxycortisone (6β,17α,21-trihydroxy-Δ^4-pregnene-3,11,20-trione). Bismuthate oxidation of one of these samples, and of authentic 6β-hydroxycortisone, yielded Zimmermann-positive products having identical chromatographic mobilities in a Bush B_5 system. A steroidal band with the same mobility as 6β-hydroxycortisone was present in the majority of urines so far studied.

Preliminary experiments, based on color reactions and identity of Rf in two systems, indicate the presence of 2α-hydroxycortisone (2α,17α,21-trihydroxy-Δ^4 pregnene-3,11,20-trione) in the urine of a young woman with adrenal carcinoma.

Urinary excretion of 6β-hydroxycortisol

Values for urinary excretion of 6β-hydroxycortisol in normal adults have been previously reported (7) to vary between 181 and 760 μg. per twenty-

four hours; in the range of 300–400 μg., the mean value is slightly higher for males than for females. The proportion is generally not greater than about 3 per cent of the normal excretion of 17-ketogenic steroids. In Table 3 are listed the values for urinary 6β-hydroxycortisol in a series of cases of Cushing's syndrome, together with those for total 17-ketogenic steroids. It may be seen that 6β-hydroxycortisol was elevated in all but 1 case (in which it was high-normal). The patient was a 52-year-old white female with Cushing's syndrome recurrent several years after a unilateral adrenalectomy. The proportion of 6β-hydroxycortisol to the total 17-ketogenic steroids in the urine also appeared to be elevated in most of the patients.

TABLE 3. URINARY 6β-HYDROXYCORTISOL IN CUSHING'S SYNDROME

Patient's sex and age	Pathologic diagnosis	Urinary steroids (mg./24 hrs.)	
		6β-Hydroxy-cortisol	17-Ketogenic Steroids
	Adrenal hyperplasia		
♀ 52	Recurrent, after unilateral adrenalectomy	0.64	31
♀ 42	Bilateral	1.20	35
♀ 45	Recurrent, after unilateral adrenalectomy	1.20	32
♀ 56	Bilateral, assoc. with bronchial carcinoma	1.86	43
♂ 55	Bilateral with pituitary chromophobe adenoma	2.52	28
♀ 44	Bilateral	2.68	29
♀ 18	Bilateral: (*a*) before treatment (*b*) after bilateral adrenalectomy; taking 40 mg. cortisol/day	4.44 3.84	25 16
♀ 65	Bilateral, assoc, with carcinoma of gall bladder	15.8	ca. 150
♀ 60	Bilateral, assoc. with oat-cell carcinoma of lung	53.2	103
	Adrenal tumors		
♀ 60	Single adrenal adenoma after removal of contralateral adrenal for multiple adenomas	3.70	25
♀ 18	Adrenal carcinoma	3.50	37
♂ 45	Metastatic adrenal carcinoma	5.12	37
♂ 46	Encapsulated adrenal carcinoma	5.92	92

Of particular interest was an 18-year-old girl with bilateral adrenal hyperplasia, whose ketogenic steroid excretion was only slightly elevated but whose 6β-hydroxycortisol excretion was roughly ten times normal. Several weeks after bilateral adrenalectomy, when she was being maintained with 40 mg. of cortisol a day, the ketogenic steroids had returned to normal, but the excretion of 6β-hydroxycortisol was still close to what it had been preoperatively, indicating that nearly 10 per cent of the administered steroid was being degraded to this single metabolite.

Evidence that corticotropin (ACTH) may stimulate production of 6β-hydroxycortisol not explainable solely as a result of increased cortisol secretion, is supplied by the studies summarized in Table 4. Two normal men, and 1 woman bilaterally adrenalectomized for Cushing's syndrome and maintained with 50 mg. of cortisol per day, were given zinc-ACTH, 40 units intramuscularly every twelve hours for two doses. Urine collec-

tions (24-hour) were begun at the time of the first injection; control urines were obtained beforehand. One of the men was taking large doses of cortisol (1.765 Gm. per day) during both the control and ACTH periods. In this subject there was more than a threefold rise in 6β-hydroxycortisol excretion, to 64.5 mg. per twenty-four hours after administration of ACTH, indicating that ACTH influenced the metabolism of the administered cortisol. An alternative explanation, that the adrenal itself was stimulated to produce this excess steroid, is difficult to accept in view of the magnitude of the output, and the results obtained in the other male subject. This man, who was not taking exogenous cortisol, excreted 4 mg. of 6β-hydroxycortisol after ACTH stimulation. This level represented an eightfold rise over the control value for 6β-hydroxycortisol, whereas 17-

TABLE 4. EFFECT OF ACTH ON URINARY 6β-HYDROXYCORTISOL

Subject: sex, age & status	Dose of exogenous cortisol/24 hrs. (mg.)	Dose of zinc-ACTH (units)	Urinary excretion (mg./24 hrs.)	
			6β-Hydroxy-cortisol	17-Ketogenic steroids
♂30, normal	1,765	0	19.5	
	1,765	40, q.12 hrs.	64.5	
♀45, bilat. adrenalectomized	50	0	0.72	
	50	40, q.12 hrs.	1.11	
♂28, normal	none	0	0.51	16.0
	none	40, q.12 hrs.	4.00	30.0

ketogenic steroid excretion only doubled during the same period. In the adrenalectomized patient also, the excretion of 6β-hydroxycortisol rose after ACTH stimulation, though to much smaller extent.

DISCUSSION

It is apparent from the data on solvent partition coefficients that the free, highly polar steroids in urine have been neglected heretofore largely because of inefficient extraction procedures. New methods, both of extraction and of chromatography, have been devised to make possible the quantitative study of these compounds. The similarity of chromatographic patterns obtained for the urine of normal subjects and of adrenalectomized patients maintained with cortisol suggests that most, if not all, of these compounds are cortisol metabolites. One of them, 6β-hydroxycortisol, appears to occupy a unique status among urinary steroids since, as already reported (7), it is quantitatively the most important unconjugated steroid in human urine.

The addition to cortisol of a 6β-hydroxyl group is a process which, as indicated by studies in which large doses of cortisol were administered

seems to take place largely at the periphery, though it may possibly occur to some extent within the adrenal gland itself. This process resembles conjugation with glucuronic acid, in that both processes render the original compound more polar, and hence more water-soluble. Renal excretion of cortisol, which is relatively insoluble unless glucuronide or other conjugation occurs, is thereby facilitated. 6β-Hydroxylation thus acts as an alternative pathway for the disposal of cortisol, though it is usually overshadowed quantitatively by the processes of A-ring reduction and subsequent glucuronide conjugation. Ulstrom *et al.* (6) have shown that in the human newborn infant, in whom these latter processes may perhaps not occur so readily, 6β-hydroxylation becomes of considerable quantitative significance.

There appear to be conditions in the adult which favor the metabolism of cortisol by 6β-hydroxylation. One of these is the third trimester of pregnancy, when not only are high urinary levels of 6β-hydroxycortisol regularly observed (a phenomenon intensified by toxemia), but administered cortisol is converted in above-normal proportions to the 6β-hydroxy derivative (7). Cushing's syndrome, whether due to bilateral adrenal hyperplasia or to adrenal tumor, is shown by the present studies to be generally associated with an elevated excretion of 6β-hydroxycortisol, and the proportion of this steroid to total ketogenic steroids is frequently, but not invariably, increased above normal. The data indicate that ACTH may be involved, perhaps through an extra-adrenal effect, in producing increased 6β-hydroxylation of both exogenous and endogenous cortisol. Explanation of the mechanisms must await further study. The physiologic action, if any, of 6β-hydroxycortisol has not been established.

The other polar steroids noted on the chromatograms would appear, from observations to date, to represent quantitatively minor products of cortisol degradation. The levels of these compounds appear to fluctuate in approximate accordance with that of 6β-hydroxycortisol. The low concentration of 6β-hydroxycortisone in relation to 6β-hydroxycortisol is interesting in view of the rough parity that exists between many of the 11-keto and 11-hydroxy metabolites of cortisol. These results suggest, among other possible explanations, that 6β-hydroxylation may inhibit oxidation at the 11-hydroxyl position.

The finding of polar steroids in the free form in human urine does not exclude the possibility that they may also be conjugated to some extent. Prior incubation of urine specimens with β-glucuronidase did not, in preliminary experiments, appear to increase the yield of 6β-hydroxycortisol. However, the hydroxyl group on carbon 6 could conceivably serve as a site for conjugation [*cf.* the finding of conjugated, 6β-hydroxy derivatives of 2α-methyl cortisol and 2α-methyl cortisone (18)], and glucuronides may

exist both of the steroid itself and of possible tetrahydro or other reduced forms.

Studies of the metabolic fate of administered steroids, by radioactive or other techniques, frequently fail to account for substantial portions of the original dose. Losses in such cases may be partly attributable to the use of techniques which fail to recover or distinguish products more polar than those anticipated.

Acknowledgments

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Addendum

At the time of submission of this manuscript, Touchstone and Blakemore (19) reported 2 patients with Cushing's syndrome and 1 patient with an adrenal adenoma (mixed clinical picture), all of whom exhibited elevated levels of urinary 6β-hydroxycortisol, as determined by another technique. In the adenoma patient, 6β-hydroxycortisol increased above the baseline value when ACTH was given.

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URINARY EXCRETION OF Δ^5-PREGNENETRIOL AND OTHER 3β-HYDROXY-Δ^5 STEROIDS BY SUBJECTS WITH AND WITHOUT ENDOCRINE DISEASE

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ABSTRACT

Δ^5-Pregnene-3β,17α,20α-triol (5-PT) was found in all urines analyzed, indicating that its presumptive adrenal precursor 3β,17α-dihydroxy-Δ^5-pregnen-20-one is regularly formed. After separation from other metabolites, 5-PT was quantitatively determined by oxidation to dehydroepiandrosterone (DHA), using periodic acid and then the Zimmermann reaction. The titers varied from 4 mg. to 53 mg. per day in 5 patients with adrenal carcinoma, and from about 0.1 mg. to 0.4 mg. in 10 subjects without endocrine disorders. Elevated titers were not found in 2 patients with Cushing's syndrome, nor in 2 with the Stein-Leventhal syndrome. Titers of 5-PT tended to vary inversely with those of DHA, suggesting the possibility that an inefficient conversion of 3β,17α-dihydroxy-Δ^5-pregnen-20-one to DHA may cause increased formation of 5-PT. Both 5-PT and DHA were sharply reduced when amphenone was given to a patient with adrenal carcinoma, whereas other $C_{19}O_2$ steroids showed little change. Thus, saturated 17-ketosteroids (*e.g.*, etiocholanolone) may arise from precursors other than DHA in adrenal cancer. Δ^5-Pregnen-3β,20α-diol was detected for the first time in the urine of all subjects without endocrine disorders. It was present and elevated in the urine of 4 of the 5 adrenal cancer patients. In adrenal carcinoma, Δ^5-androstenediol excretion was about one-tenth that of the DHA. This steroid was also found in the urine of 3 other subjects.

THE occurrence of numerous 3β-hydroxy-Δ^5 steroids in the urine of persons with adrenocortical carcinoma has long been known. Their abundance has been ascribed to an insufficiency of adrenal 3β-hydroxysteroid-dehydrogenase relative to the large production of Δ^5-pregnenolone, dehydroepiandrosterone (DHA), and related compounds[1] by the neoplastic adrenocortical tissue (1).

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[1] The following terms are used in the text:

5-PT is Δ^5-pregnen-3β,17α,20α-triol.

20β-5-PT is Δ^5-pregnen-3β,17α,20β-triol.

In normal persons, DHA has been the only such steroid found in urine in significant amount. Identification of DHA in the adrenal venous blood of 3 women by Hirschmann *et al.* (2) has demonstrated its adrenocortical origin. The importance of DHA has been emphasized by VandeWiele and Lieberman (3) in experiments indicating it to be a major adrenal "androgen," as well as the source of the urinary androsterone and etiocholanolone from the normal adrenal cortex. The precursor of DHA in an adrenal

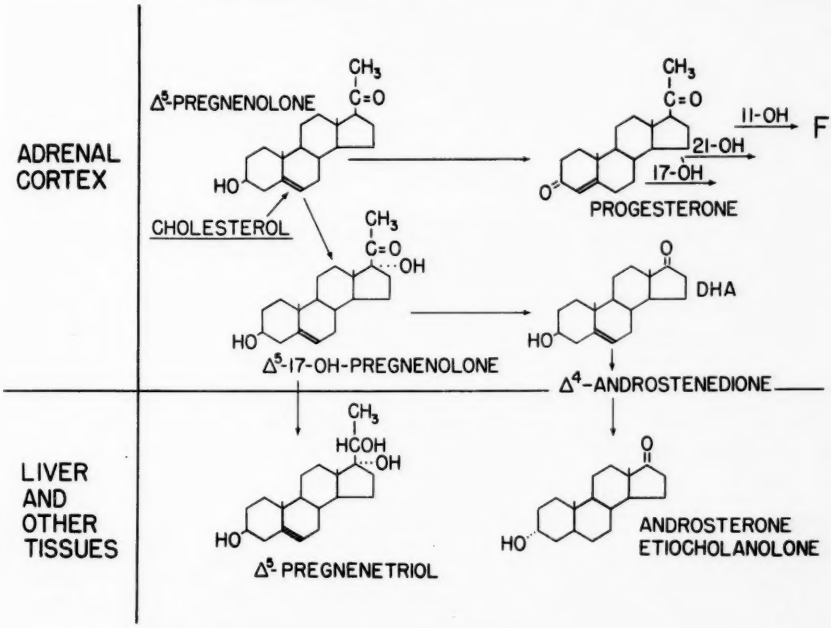

FIG. 1. Suggested pathway for the formation of 17-hydroxypregnenolone and DHA in the adrenal cortex, and their urinary excretory products (3). The 2 isomeric metabolites of DHA are indicated by one structural formula. Androsterone has the 5α-H configuration and etiocholanolone has the 5β-H configuration.

carcinoma has been shown to be 17-hydroxypregnenolone (4). Figure 1 illustrates this reaction sequence.

If the latter reaction also occurs in the normal adrenal cortex, the presumptive precursor of DHA, androsterone and etiocholanolone, *viz* 17-hydroxypregnenolone, must be regularly synthesized in substantial

Pregnenolone, or Δ⁵-pregnenolone, is 3β-hydroxy-Δ⁵-pregnen-20-one.
17-Hydroxypregnenolone is 3β,17α-dihydroxy-Δ⁵-pregnen-20-one.
DHA, or dehydroepiandrosterone, is 3β-hydroxy-Δ⁵-androsten-17-one.
Androstenediol is Δ⁵-androsten-3β,17β-diol.
Pregnanetriol is pregnane-3α,17α,20α-triol.

amounts. Some part of this steroid would then be expected to escape into the bloodstream unchanged, and be excreted as the metabolite Δ^5-pregnene-3β,17α,20α-triol (5-PT). Urinary 5-PT would then provide direct evidence for the formation of 17-hydroxypregnenolone, and the amount of 5-PT would reflect the amount of 17-hydroxypregnenolone which had not been further transformed. Previous workers have identified 5-PT in the urine of adrenal carcinoma patients (5–7), normal and hypertensive subjects (8–10), and 2 patients with adrenal hyperplasia (7).

By the same reasoning, urinary DHA, plus its metabolite androstenediol (11) serves as an index of the amount of DHA which has not been further converted, and urinary pregnenediol probably reflects the amount of pregnenolone which was secreted before being transformed (12, 13).

The present paper gives techniques for the determination of urinary 5-PT, and correlates the excretion of 5-PT with that of DHA in a variety of subjects. To see whether a precursor-product relationship could be shown, the specific activities of 5-PT and DHA were compared on four consecutive days after the administration of 4-C^{14} cholesterol to a boy with an adrenal carcinoma. Another metabolite, pregnenediol, was regularly found to be present and was quantitatively determined in some cases. In 1 adrenal carcinoma patient the effect of amphenone on these 3β-OH-Δ^5 steroids was followed and compared with its effect on urinary $C_{19}O_2$ steroids. After oral ingestion of 17-hydroxypregnenolone, 5-PT was sought as a metabolite.

MATERIALS AND METHODS

Subjects

Ten subjects without endocrine abnormalities, 6 subjects with various endocrine abnormalities, and 5 subjects with adrenocortical carcinoma were studied.

Reagents

Ether: Baker and Adamson, reagent grade, washed free of peroxides with a saturated solution of ferrous sulfate prior to use. When precipitating digitonides, Mallinckrodt "Ether for Anesthesia" was used. This ether should be redistilled if more than 100–200 ml. is required.

Digitonin: Obtained from Eimer and Amend, New York, N. Y.

Methanol: "Spectroquality"; Matheson, Coleman and Bell, East Rutherford, N. J.

Other reagents and their preparation have been previously described (14–16).

Equipment

Spectrophotometer: Cary, model 14 recording spectrophotometer; Applied Physics Corp., Pasadena, Calif.

Scintillation counter: Packard tri-carb liquid scintillation spectrometer; Packard Instrument Co., La Grange, Illinois.

Steroid reference compounds[2]

Authentic samples of most of the steroids determined were available. Δ⁵-Pregnen-3β,17α,20β-triol was prepared from 3β,17α-dihydroxy-Δ⁵-pregnen-20-one by reduction with potassium borohydride, and crystalline Δ⁵-pregnen-3β,17α,20α-triol was isolated from pooled extracts of urine from several patients with adrenocortical carcinoma. These preparations have been described in detail (17).

Hydrolysis of steroid conjugates and their extraction from urine

Urine collections covering twenty-four hours were treated as previously described (15). Briefly, the urine was incubated with β-glucuronidase for seventy-two hours, then acidified to pH 1.0, and continuously subjected to extraction with ether for forty-eight hours. The extraction was repeated after acidification of the urine to pH 0. In order to avoid decomposition of free steroids by prolonged exposure to heat and acid, the extracts in the receiving flasks were removed each morning and evening and replaced with fresh ether. Neutral fractions of each extract were combined.

Partition column chromatography

Aliquots of each extract were chromatographed on a partition column modified from that described earlier (14–16) in order to resolve larger amounts of extract. Fifteen grams of the silica-alumina catalyst, in a stationary phase of 50 per cent v/v aqueous ethanol, were packed into a 25×36 mm. tube. Compositions and volumes of the mobile solvents, designed to secure the maximal possible separation of the expected 3β-OH-Δ⁵ steroids, were as follows:

Solvent A (100 per cent hexane)
Eluate A1, 0–25 ml., plus the pore volume of about 10 ml., making 35 ml. in all.
Eluate A2, 25–150 ml., containing all the DHA and most of the pregnenediol.
Solvent B (3 per cent chloroform; 97 per cent hexane)
Eluate B1, 0–140 ml., containing all the androstenediol and 17-hydroxypregnenolone, if present.
Eluate B2, 140–250 ml., containing pregnane-3α,17α,20α-triol.
Solvent C (8 per cent chloroform; 92 per cent hexane)
Eluate C1, 0–140 ml., containing the 5-PT.
Eluate C2, 140–360 ml.
Further solvents used to elute more polar compounds will be described elsewhere.

Comment. This column will fractionate extracts representing the urine collected for 0.25–0.50 day from subjects with no overproduction of steroids, and for 0.05 to 0.10 day from subjects with adrenal carcinoma. If the column is moderately overloaded, it will still provide good separations, but part or all of any one metabolite may appear in a later eluate than expected.

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[2] Dehydroepiandrosterone was obtained from Chemed Inc., White Plains, N. Y.; Δ⁵-androsten-3β,17β-diol from Ciba Pharmaceutical Products, Summit, N. J.; and 3β,17α-dihydroxy-Δ⁵-pregnen-20-one from Mann Research Laboratories, 136 Liberty St., New York 6, N. Y. Samples of crystalline pregnane-3α-17α,20α-triol, Δ⁵-pregnen-3β,20α-diol and Δ⁵-pregnen-3β,20β-diol were generously given by Dr. J. J. Schneider, Jefferson Medical College, Philadelphia.

Precipitation of 3β-OH steroids with digitonin

The dried fractions, in glass-stoppered centrifuge tubes, were dissolved in 85 per cent ethanol containing 10 mg. of digitonin per ml., using 1 ml. per mg. of estimated 3β-OH steroid. After standing for sixteen to eighteen hours, 10 volumes of ether (Mallinckrodt, for anesthesia) were added with mixing. When the digitonides had flocculated, the mixture was centrifuged, the supernatant "alpha" fraction was decanted, and the ether flocculation of the precipitate was repeated.

The digitonides were decomposed twice, by heating the first time to 60°, and the second time to 80° C. for thirty minutes with 1 ml. of anhydrous pyridine per each ml. of digitonin solution used initially, precipitating with ether and decanting each time. The combined ethereal supernatant solutions were washed three times with one-tenth volume of 10 per cent aqueous sulfuric acid and twice with water, in order to remove all pyridine and digitonin. After drying with anhydrous sodium sulfate the ether was filtered and evaporated to dryness. Inadequate washing resulted in a re-formation of 5-PT digitonide during evaporation of the solvents.

This procedure resulted in complete precipitation in model experiments with DHA, 17-hydroxypregnenolone and 20β-5-PT. Interestingly, 3β,11β-dihydroxyandrostane-17-one was not precipitated. The decomposition procedure appeared to be adequate, as a third treatment yielded no further 3β-OH steroid.

Paper chromatography

Washing of paper. Sheets of Whatman #1 and #3MM paper were cut into 15×57 cm. strips and washed by descending capillary flow for three days with 2 *N* acetic acid, three days with water, and five days with absolute ethanol. To avoid siphoning of the washes, only one sheet of paper was used per trough.

Solvent systems. Table 1 shows the compositions of the solvent systems and the times of development for separation of the compounds of interest.

TABLE 1. PAPER CHROMATOGRAPHY OF 3β-OH-Δ^5 STEROIDS

Column eluate	Solvent systems						Steroid**	Mobility† (cm.)
	Code	Hours*	Ligorin	Toluene	Methanol	Water		
A2	A-1	8	100	—	90	10	Pregnenediol DHA Epiandrosterone	8–11 18–22 24–27
	A-2	4	100	—	70	30	Androstenediol Pregnenediol DHA	6– 8 12–14 20–23
B1	B-1	3	50	50	70	30	Androstenediol Pregnenediol	15–22 27–31
C1 C2‡	B-1	6	50	50	70	30	5-PT Cpd. B§ 20β-OH-5-PT	24–28 25–28 34–40

* Running times are for Whatman 3 MM paper. Mobilities on No. 1 paper are approximately one-third less.

** See text for chemical names of these steroids.

† Approximate locations.

‡ 5-PT may be largely in eluate C2 if the column is overloaded, or with extracts of urine from some subjects with adrenal carcinoma.

§ Compound B (corticosterone) serves as a good marker for 5-PT.

Spot tests on paper chromatograms. Narrow strips were tested by over-running with the Allen reagent (18) which produces an immediate strong reaction with all these 3β-OH-Δ⁵ steroids (17), by the Zimmermann reaction for 17-ketosteroids, and in the case of the C eluates, by the alkaline triphenyltetrazolium chloride reduction test for α-ketols.

Pure reference samples of the compounds of interest were always chromatographed on parallel strips, except for 5-PT, which was not available in sufficient amount. Corticosterone was found to have the same mobility as 5-PT in the solvent system used. Accordingly both corticosterone and 20β-5PT were chromatographed in parallel with 5-PT containing fractions.

Elution. Reactive areas cut out from chromatograms were eluted with "spectro-quality" methanol by descending capillary flow. The dried residue was redissolved in chloroform (previously washed with sulfuric acid (14)) and washed once with one-twentieth volume of 0.2 *N* sodium hydroxide, once with 0.02 *N* acetic acid in 25 per cent sodium chloride, and twice with water. After drying the chloroform with a little anhydrous sodium sulfate, it was evaporated to dryness. All glassware for the elution and washing steps had been cleaned in chromic-sulfuric acid cleaning solution to which had been added one-fourth volume of concentrated nitric acid.

If the concentration of 3β-OH-Δ⁵ steroid was not sufficiently high, it was necessary to remove contaminants before assay. Eluted bands containing DHA, androstenediol and 5-PT were combined and re-run on a single partition column which again separated them in a greatly purified state. The pregnenediol fractions were separately re-run, as this metabolite is not well separated from DHA on the column.

Quantitative assays

When amounts sufficed, all four 3β-OH-Δ⁵ steroids were identified and assayed by means of their characteristic spectra and absorption peaks in the Allen reagent at room temperature (17). Smaller amounts were visually estimated from the size and intensity of the spot tests. DHA was also determined by the micro Zimmermann reaction (19).

The 5-PT fractions were usually also oxidized with periodic acid (20). The resulting DHA was then extracted with methylene chloride, and quantitated by the foregoing two procedures. A third aliquot was chromatographed on paper in parallel with a sample of authentic DHA.

Metabolism of 3β,17α-dihydroxy-Δ⁵-pregnen-20-one by a normal male

A suspension of 100 mg. of the crystalline steroid in water was ingested. The residue in the vessel was dissolved in ethanol, then diluted with water, and ingested as an opalescent solution. Urine was collected over the succeeding 24-hour period.

RESULTS

Bands seen on paper chromatograms

The four 3β-OH-Δ⁵ steroids being sought were frequently the only reactive substances on their respective paper chromatograms. In column eluate A2, pregnenediol and DHA were clearly visible with the Allen test (17) and well separated. Pregnenediol was always detected opposite the Δ⁵-pregnene-3β,20α-diol reference standard, well separated from the 20β-OH isomer, indicating the urinary compound to be the 20α-OH isomer. Two extracts from the urine of adrenal carcinoma patients exhibited an unidentified material near the origin, also reacting in the Allen test.

Occasionally a weak Zimmermann reaction corresponding to epiandrosterone was seen. In eluate B1, a further small amount of pregnenediol sometimes appeared, as well as a reaction opposite androstenediol in the instances in which it was sought. In the 5-PT-containing fraction not more than a trace of other material was detected on the papergrams, except in 2 of the cases of adrenal carcinoma. When aliquots of the eluted 5-PT bands oxidized with HIO_4 were chromatographed on paper, DHA was found as the only product, as judged by mobility and color tests.

Adrenocortical carcinoma

5-PT. As shown in Table 2, all 5 subjects excreted substantial amounts of 5-PT—in one reaching more than 40 mg. per day. A fairly good correlation

TABLE 2. URINARY EXCRETION (MG./24 HRS.) OF FOUR 3β-OH-Δ⁵ STEROIDS IN PATIENTS WITH ADRENAL CARCINOMA

Subject	Sex & age	5-PT		DHA	Androstenediol	DHA + androstenediol	Pregnenediol
		Allen*	HIO_4-KS				
L.S.	F 55	4.3	3.7	16.2	0.75†	17.0	2.0†
V.F.	F 45	5.5	5.1	11.7	1.3	13.0	4.1
T.R.	M 59	4.8	4.8	23.0	2.6	25.6	1.4
R.S.	M 30	8.0	6.3	18.0	0.3†	18.3	1.8
D.A.‡	M 8	28.2 44.5 30.5 16.2	20.0 53.0 30.2 16.9	5.2 4.0 6.8 11.8	0.4†	5.6	n.f.§ n.f.§

* The corrected height of the peak at 530 mμ in the reagent containing 77.5% sulfuric acid was used for quantitation (17).
† Visually estimated from spot tests.
‡ Four consecutive 24-hour urine collections were analyzed.
§ Metabolite not found by the techniques employed.

between the two methods of assay was obtained (Table 2). The spectra in the 77.5% Allen reagent at room temperature (17) were almost identical with those of the pure isomeric 20β-5-PT (Fig. 2). The absorption spectrum after oxidation with periodic acid showed the product to be DHA (Fig. 3).

The isomeric 20β-5-PT reported by Fukushima *et al.* (21) to be excreted in amounts comparable to those of the 20α-OH isomer was never found in our studies. Only very small amounts could have gone undetected. None of the adrenal carcinoma subjects excreted 17-hydroxypregnenolone insofar as could be determined.

DHA and Δ⁵-androstene-3β,17β-diol. As expected, DHA excretion was elevated in all these subjects (Table 2). As shown in the table, androstenediol, a metabolite of DHA, did not comprise more than about 10 per cent of the sum of both steroids.

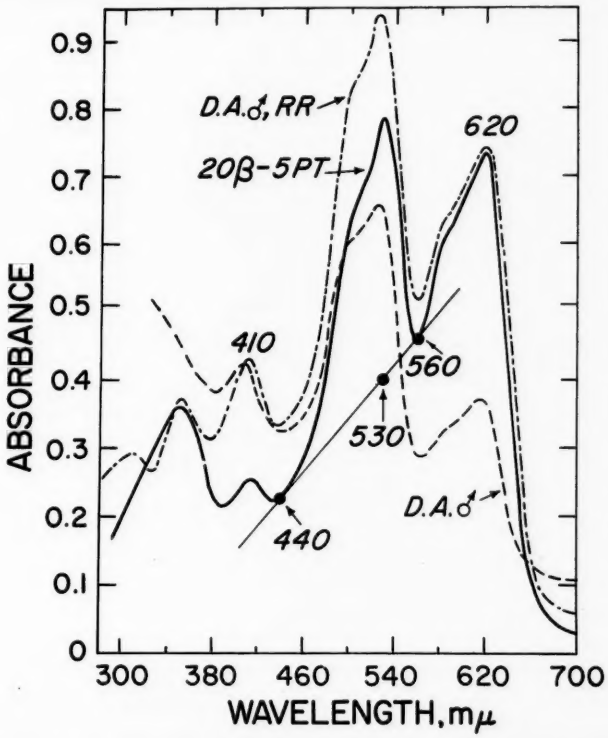

FIG. 2. Absorption spectra in the Allen reagent (77.5% sulfuric acid) after one hour at room temperature. The solid line is pure Δ⁵-pregnene-3β,17α,20β-triol (20β-5PT); the dashed line is a washed eluate from the final paper chromatogram, 5-PT band; and the dot-dash line shows the improved spectrum after this eluate was re-run (RR) on a partition column. The graphic method of estimating the corrected absorbance at 530 mμ is illustrated (17).

Δ⁵-Pregnene-3β,20α-diol. Four of the 5 adrenal carcinoma patients excreted pregnenediol in amounts between 1.4 and 4.1 mg. per day, whereas it was not detected in the urine of the fifth subject. In Figure 4 the absorption spectrum of a urinary fraction is compared with that of the pure compound. These findings indicate that pregnenediol is frequently an important 3β-OH-Δ⁵ steroid in adrenal carcinoma. The amount in the urine reflects the portion of pregnenolone which escapes conversion to other biosynthetic intermediates.

Correlations. It is clear that there was no positive correlation between the amounts of 5-PT and DHA excreted by any one subject. In fact, the highest levels of 5-PT were accompanied by the lowest levels of DHA (Subject D. A.). The wide variation in the day-to-day titers in Subject

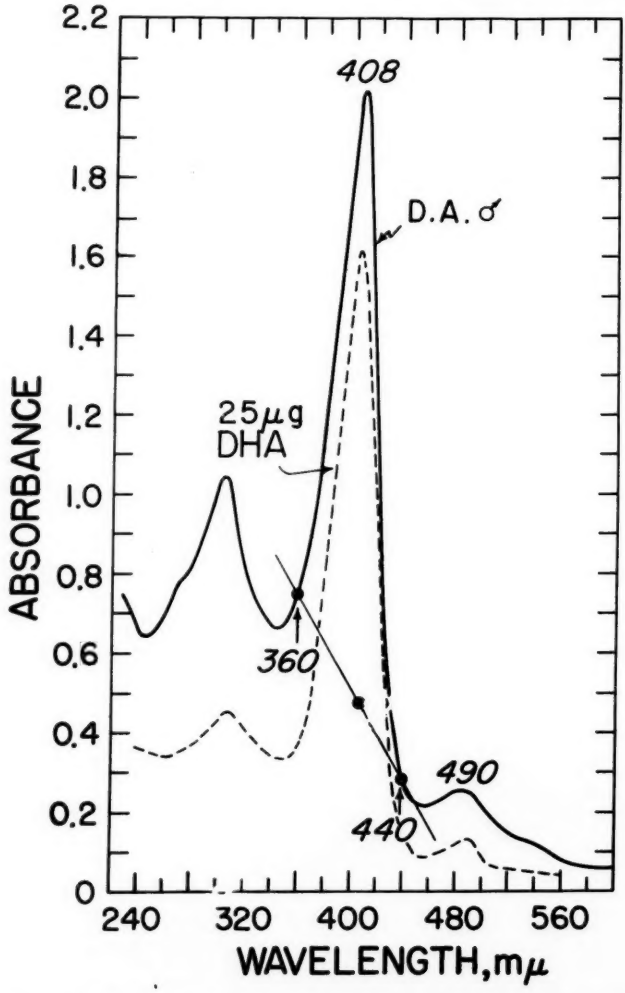

FIG. 3. Absorption spectrum of the product when a purified 5-PT fraction was oxidized with periodic acid and extracted. Dehydroisoandrosterone, the oxidation product (dashed line), shows a peak at 490 mμ when carried through the oxidation procedure. The graphic method of estimating the corrected absorbance at 408 mμ is illustrated (17).

D. A. is of interest. Here again the trend is towards an inverse relationship between 5-PT and DHA.

Relative specific activities of 5-PT and DHA. Subject D. A. was given 50 μC, or 7.2×10^7 c.p.m. of 4-C^{14} cholesterol (specific activity 16.5 μC per mg.) intravenously. Specific activities in c.p.m. per μmole of the isolated

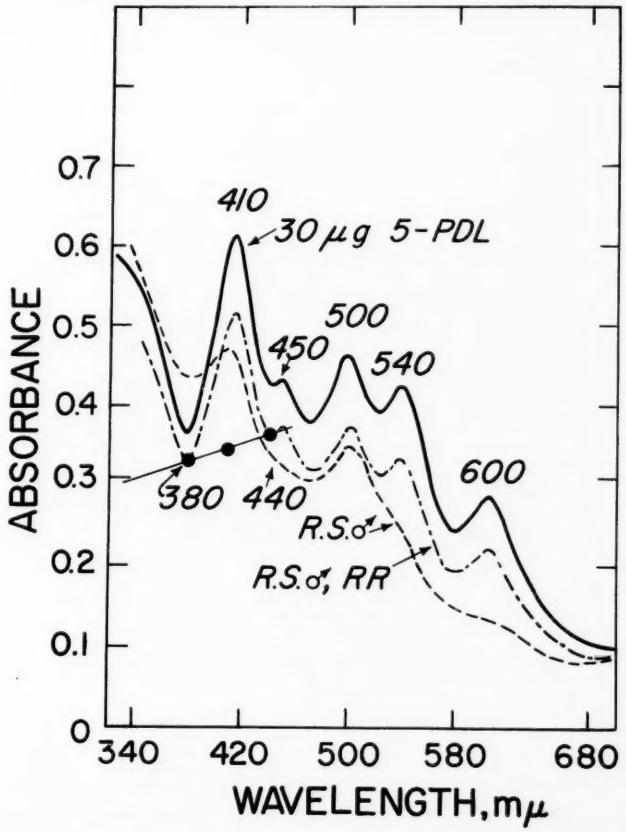

FIG. 4. Absorption spectra in the Allen reagent after thirty minutes at room temperature. The solid line is pure Δ⁵-pregnene-3β,20α-diol (5-PDL); the dashed line is a washed eluate from the final paper chromatogram, pregnenediol band; and the dot-dash line shows the improved spectrum after this eluate was re-run (RR) on a partition column. The graphic method of estimating the corrected absorbance at 410 mμ is illustrated (17).

5-PT and DHA were determined for the four succeeding 24-hour urine collections. Determinations on the original column eluates, 3β-OH fraction, agreed closely with those on the eluted papergram bands. Table 3 shows that the specific activities were still rising after four days, indicating that equilibrium had not been reached, and that the C^{14} cholesterol was slowly converted to steroids, the maximum available concentration being reached only after four days. The changes in specific activities of the two steroids were roughly parallel. (The value for DHA on day 2 may be in error.) There were no important differences between the activities of the two

TABLE 3. SPECIFIC ACTIVITIES OF 5-PT AND DHA ISOLATED FROM THE URINE OF A BOY (SUBJECT D.A.) WITH ADRENAL CARCINOMA DURING FOUR DAYS AFTER THE INFUSION OF 4-C^{14} CHOLESTEROL

C.p.m. per μmole	Day			
	1	2	3	4
5-PT	58.4	162.0	210.0	241.0
DHA	89.5	98.0	207.0	227.5

metabolites. The small differences which were observed were in favor of DHA, with 5-PT increasing proportionately from 65 per cent of DHA on day 1 to 98 per cent on day 3. Although no valid conclusions can be drawn as to whether 17-hydroxypregnenolone was a precursor of DHA, the similarity in specific activities at three and four days indicates that this could have been the case.

Effect of amphenone on 3β-OH-Δ^5 steroids. In another patient with adrenal carcinoma, several 3β-OH-Δ^5 steroids were determined before, during and after treatment with amphenone. Analyses showing depression by amphenone of corticosteroid metabolites in this subject (AC) have been published (14). The results in Table 4 show that 5-PT, DHA and (pregnenediol +pregnanediol) were all sharply suppressed by amphenone.

Values for the total ($C_{19}O+C_{19}O_2$) fraction obtained by CrO_3-KS assays (22) are also listed. Paper chromatography showed that these titers reflect chiefly DHA, etiocholanolone and $\Delta^{3,5}$-androstadiene-17-one, whereas androsterone was not seen (14). It is noteworthy that the small decline in this fraction produced by amphenone was accounted for by the fall in DHA, and that on withdrawal of the drug these titers rose sharply above control levels, whereas 5-PT and DHA showed much smaller increases.

In this study, therefore, 5-PT and DHA varied in parallel, but etiocholanolone changed independently of both. Thus, much of the etiocholanolone being measured apparently did not originate from 17-hydroxy-

TABLE 4. EFFECT OF AMPHENONE ON THE 3β-OH-Δ^5 URINARY STEROIDS (MG./24 HRS.) OF A PATIENT WITH ADRENOCORTICAL CARCINOMA (SUBJECT AC)*

Period	5-PT†	DHA	Pregnenediol‡ +pregnanediol	$C_{19}O_2$§
Control	8.2	6.0	8.8	26.4
Amphenone	0.7	0.7	3.7	22.9
Post-amphenone	2.8	4.0	3.3	73.8

* First neutral extract only, prepared by continuous extraction at pH 0.8 after hydrolysis with β-glucuronidase.

† Determined by the HIO_4-KS method (23). The chief oxidation product was DHA, shown by paper chromatography.

‡ Pregnane-3α,20α-diol and Δ^5-pregnen-3β,20α-diol were not separated.

§ Titers on the total $C_{19}O+C_{19}O_2$ eluates from the partition column, using the CrO_3-KS assay (14, 22).

pregnenolone. The relation of DHA to etiocholanolone and androsterone described by VandeWiele and Lieberman (3) does not hold in adrenal carcinoma (37).

Subjects without endocrine abnormalities

5-PT. This metabolite was found in the urine of all 10 subjects tested. Improved techniques detected 5-PT in several urines in which it was first thought to be absent (23). Table 5 shows that most values were near 0.1–0.2 mg. per day, though in 1 healthy normal male and in 1 female the values were over 0.4 mg.

TABLE 5. URINARY EXCRETION OF 3β-OH-Δ⁵ STEROIDS (MG./24 HRS.) IN SUBJECTS WITHOUT ENDOCRINE ABNORMALITIES

Subject	Sex & age	5-PT		DHA	Androstenediol	Pregnenediol*
		Allen	HIO_4-KS			
L.S.-1	M 28		0.44	3.4	0.50*	0.50
L.S.-2**			1.10	3.3		
J.B.†	M 26		0.10*	0.90	present	
L.B.	F 38	0.45	0.36	0.12	0	0.18
L.A.	F 22	0.10*		n.f.‡		0.20
B.U.	F 24	0.20*		0.20*		0.30
E.K.	F 51		0.22§	0.18*		0.08
K.R.	F 49		0.10			
M.D.	F 59		0.04	Tr‖		0.12
H.C.†	F 52	Tr‖		n.f.§		0.15
S.R.	F 6		0.07	0.1*		present

* Estimated visually from size and intensity of spot tests.
** Urine collected over 24 hours following oral ingestion of 100 mg. of 17-hydroxypregnenolone.
† First neutral fraction only (pH 0.8).
‡ Compound was not detected by the techniques employed.
§ After hypophysectomy and subsequent infusion of ACTH (15).
‖ Trace.

Δ⁵-Pregnen-3β,17α-diol-20-one. Although the origin of 5-PT from 17-hydroxypregnenolone in man seems beyond doubt, it has not as yet been experimentally demonstrated. Rabbit skeletal muscle has been shown to effect this conversion (24).

Accordingly a 100-mg. dose of 17-hydroxypregnenolone was orally administered to Subject L.S. The succeeding 24-hour urine yielded 1.10 mg. of 5-PT, an increase of only 0.66 mg. over the control collection (Table 5). Poor absorption of this difficultly soluble steroid may account for the low recovery. These findings therefore only suggest that the conversion of 17-hydroxypregnenolone to 5-PT had occurred. There was no change in DHA excretion.

DHA. The excretion of DHA was markedly higher in 2 normal males than in females of similar age. No DHA could be found in the urine of some

of the women (Table 5). Three subjects (2 males and 1 female) were tested for androstenediol; the results were positive only in the 2 normal males.

The only deduction that can be made regarding a relationship between 5-PT and DHA excretion in this group of subjects is that a slight rise in the excretion of either metabolite is not necessarily accompanied by a rise in the other.

Δ^5-Pregnen-3β-20α-diol. Small amounts of this metabolite were also detected in the urine of every subject in this category in whom it was sought—even in 2 cases in which DHA was not found. Precise location of the band opposite the reference standard and a characteristic sequence of colors developed with the Allen reagent permitted identification with con-

TABLE 6. URINARY EXCRETION OF 3β-OH-Δ^5 STEROIDS (MG./24 HRS.) IN SUBJECTS WITH VARIOUS ENDOCRINE ABNORMALITIES

Subject	Sex & age	Diagnosis	5-PT		DHA	Androstenediol*	Pregnenediol*
			Allen	HIO_4-KS			
T.A.	F 49	Cushing's disease	0.18*		0.24*	0	0.40
O.H.	F 55		0.25	0.26	0.30	0.08	0.25
J.R.	F 21	Stein-Leventhal syndrome	0.13	0.18	0.40	0.05	0.15
S.H.	F 16		0.12*	0.16	0.82	0	0
G.M.	15	Hermaphrodite	0.25*		0.24		
L.H.	F 6	Precocious sexual development	present		n.f.†		present

* Estimated visually from size and intensity of spot tests.
† Compound not detected by techniques employed.

siderable certainty. Table 5 shows that most excretion levels were near 0.1–0.2 mg. per day, though about 0.5 mg. per day was found in Subject L.S. These results indicate that a small amount of pregnenolone regularly escapes conversion in the adrenal cortex, and possibly also in the gonads.

Subjects with various endocrine abnormalities

In Table 6 it is seen that, at most, there was a very small elevation in the excretion of the four 3β-OH-Δ^5 metabolites in 2 women with Cushing's disease. In 2 women with proven Stein-Leventhal syndrome, the titers of DHA were higher than in the controls listed in Table 5. The excretion of 5-PT was not elevated, and in 1 case pregnenediol was not found.

DISCUSSION

The uniform occurrence of 5-PT in the urine of all the subjects studied is evidence that 17-hydroxypregnenolone is regularly formed in the adrenal cortex. It is also possible that this steroid may be synthesized by testicular

or ovarian tissue. Thus, as has been discussed by others (4, 25, 26), 17-hydroxypregnenolone can well be an important precursor of dehydroepiandrosterone as well as of other adrenocortical steroids.

The validity of the determinations of 5-PT is dependent upon the specificity and accuracy of the analyses. Our procedure effected isolation from other metabolites for all practical purposes. Quantitation by determining the entire absorption spectrum in the Allen reagent, then calculating from the peak at 530 mμ, also provided confirmatory identification. Determination after conversion to DHA by periodic acid, with chromatographic and spectrographic proof that DHA was virtually the only Zimmermann reactant being measured, would appear to be an entirely specific procedure.

The acetaldehydrogenic procedure of Cox (7), though doubtless advantageous in many respects, requires absolute separation from other reactants before any one such product can be specifically determined. In separating 5-PT from pregnanetriol and its 3α-hydroxy isomers, digitonin precipitation is most valuable. Moreover, both the partition column and the paper chromatographic system used in this study separate 5-PT from pregnanetriol. It is obvious from the absorption spectra either in sulfuric acid or in the Allen reagent (17) that the presence of either metabolite will lead to overestimation of the other by these techniques.

Since the 3β-OH-Δ⁵ steroids were expected to be conjugated largely as sulfates, our hydrolytic procedures were designed to secure maximal hydrolysis of steroid sulfates with minimal decomposition. This procedure is known to furnish good yields of DHA (27). However, in view of the extensive purification of 5-PT, our excretion values are probably minimal. Yet our titers for normal persons, 0.1–0.4 mg. per day, are higher than some others in the literature [Fotherby (9), 0.035 mg. per liter; Cox (7), 0.1 and 0.08 mg. per day]. Nowaczynski *et al.* (10) reported a normal mean excretion of 0.293 mg. per day. Excretion of 5-PT by normal persons is usually much less than that of pregnanetriol, in our experience.

Our data on the relative specific activities of urinary 5-PT and DHA after administration of 4-C^{14} cholesterol provide only suggestive information regarding the origin of DHA from 17-hydroxypregnenolone. The fact that levels of 5-PT and DHA do not vary in parallel, and even seem to be inversely related, may be a reflection of the relative activities of various enzyme systems acting on 17-hydroxypregnenolone. Thus, if its conversion to DHA is inefficient, urinary 5-PT may increase while DHA decreases.

Amphenone, studied in 1 adrenal carcinoma patient, depressed 5-PT excretion to roughly the same marked extent as previously shown for DHA (28, 29). The fact that the major $C_{19}O_2$ steroid, etiocholanolone, was not simultaneously depressed, and was increased to a much greater extent on withdrawal of the drug, suggests that much of the etiocholanolone did not originate from DHA in this instance (3).

Although 17-hydroxypregnenolone has been found in the urine of a boy with an adrenocortical tumor (30), it was not detected in any of our subjects. This indicates that reduction of unchanged 17-hydroxypregnenolone to 5-PT was complete in these cases.

The finding of pregnenediol in most adrenal tumor patients was not unexpected, as it had previously been isolated from the urine of 2 such patients, as well as from the urine of a girl with virilism and a patient with adrenal hyperplasia (12, 31, 32). In the 1 adrenal carcinoma patient (Table 2) whose urine yielded negative results, only about 500 μg. per day could have escaped detection.

In the urine of normal persons, pregnenediol has not previously been reported. Our tests show it to be a frequent constituent, reflecting the transitory existence of pregnenolone. Moreover, androstenediol was also detected in the urine of 3 subjects without adrenocortical tumors.

The 2 subjects with Cushing's disease contrast sharply with the adrenal tumor patients, in that there was no real elevation of the excretion of 3β-OH-Δ^5 metabolites. Both patients exhibited a twofold to threefold elevation of cortisol metabolites. Low titers for DHA in Cushing's syndrome have previously been noted by Jailer *et al.* (33). It can thus be deduced that overproduction of cortisol, with the necessarily increased amounts of pregnenolone involved, does not always result in elevated excretion of pregnenediol, 5-PT or DHA. High titers in cases of adrenal tumor must therefore be due to differences in the synthetic and metabolic activities of the neoplastic tissue itself.

Neither pregnenediol nor 5-PT excretion was elevated in the 2 girls with the Stein-Leventhal syndrome, though the urine of both contained abnormally high amounts of androgen metabolites, and in Subject J. R., of cortisol metabolites. However, DHA excretion was definitely higher than in the other women in our series. Other workers have found a normal (34) or slightly elevated (35) excretion of DHA in the Stein-Leventhal syndrome, and Cox and Shearman (36) have reported that 5-PT excretion is high in such subjects when assays are made by their procedure. Perhaps greater uniformity in analytical methods or classification of subjects is needed to clarify this situation.

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A SIMPLIFIED PROCEDURE FOR DETERMINATION OF URINARY DEHYDROEPIANDROSTERONE*

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ABSTRACT

When urinary steroids were hydrolyzed by hot-acid procedures, the values for Pettenkofer chromogens in the ketonic fraction did not agree with the values for dehydroepiandrosterone as determined by the Fotherby method. Values for urinary dehydroepiandrosterone determined by measuring the Pettenkofer-reacting material in ketonic extracts of urine after hydrolysis by the two-phase solvolysis procedure of Burstein and Lieberman agreed well with those determined by the chromatographic method of Fotherby. The same results were also obtained in the ketonic extracts of urines prepared by the author's procedure. The latter method is easier than the chromatographic techniques and shorter than the solvolysis method. Thus it can be recommended as a useful procedure for the routine clinical determination of dehydroepiandrosterone excretion.

THE problem of the formation of artifacts during the hydrolysis of urinary steroids is particularly important in the determination of dehydroepiandrosterone (DHA). If such artifacts could be avoided or minimized, the study of the metabolism of steroids might be materially advanced.

The principal conjugate of DHA in plasma and urine is the sulfate. After chemical or enzymatic hydrolysis, urinary DHA at present is estimated by some modification (1, 2) of the Zimmermann reaction (3), or by the reactions of Patterson (4), Allen *et al.* (5) or Pettenkofer (6). Usually the urinary extracts are purified by means of the Girard T reagent or by a chromatographic technique (7–10) but sometimes, undesirable though it is, the color reaction has been carried out on the total neutral lipid fraction, without any further purification.

Frequently the values for urinary Pettenkofer chromogens have been considered to represent the daily output of DHA. Although it has been found that these chromogens are an index of adrenal activity, their levels in urinary neutral ketonic extracts after hot-acid hydrolysis should not be reported as dehydroepiandrosterone (11, 12).

In a previous paper (12) the author studied the Pettenkofer chromogen

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content of the total ketonic, and alpha and beta ketonic fractions in the urines of healthy persons and patients with various endocrine disorders. These urinary extracts were obtained after routine hot-acid hydrolysis (13). Because of the nature of the results, we decided to study the Zimmermann and Pettenkofer chromogens in extracts of urines obtained after hydrolysis and extraction by 4 different procedures, and to compare the findings with those obtained by the chromatographic method of Fotherby (14). The Fotherby procedure is one of the more reliable current methods for the determination of DHA. However, along with the other chromatographic methods it is rather lengthy for routine laboratory use.

METHODS

Hydrolysis, Extraction and Purification of Urinary Steroids

No preservatives were added to the urine, but it was kept at 4° C. As a rule, extraction was undertaken one or two days after collection but in every case no more than a week elapsed between collection and extraction.

Method A. The urinary steroids were acid-hydrolyzed and extracted with chloroform by the continuous extraction procedure of Dorfman and Shipley (15). The final chloroform extracts were evaporated to dryness under reduced pressure. The residue was dissolved in ether and the ether extract was treated as in method B.

Method B. The urinary steroids were hydrolyzed and extracted with ether, and the ether extracts washed with 2 *N* sodium hydroxide and water as described by Pincus (13). The ether extracts were dried.

Method C. The two-phase solvolysis method of Burstein and Lieberman (16) was used. The final ether extract was treated as in method B.

Method D. The sample of urine was refluxed for six hours at 100° C. (in a boiling water bath) without adjustment of the pH. (The level of urine in the flask must not be above the level of the water in the bath so that heating is uniform.) After cooling, the steroids were extracted with ether and treated as in method B.

Method E. For determination of DHA, the chromatographic method of Fotherby (14) was used. Only the Pettenkofer reaction was carried out on the dry residue obtained after chromatography.

Girard reaction. The dry ether extracts obtained by methods A, B, C and D were submitted to purification by means of the Girard T reagent (17).

Color reactions. The ketonic fraction was dissolved in ethanol, and Zimmermann (13) or Pettenkofer (6) reactions were carried out in the usual manner (12).

RESULTS

Table 1 shows the excretion of DHA and Pettenkofer chromogens in 27 subjects[1]—10 normal females, 7 normal males, and 10 patients with various endocrine disorders. DHA values were determined by the chromatographic method of Fotherby (E). Pettenkofer chromogens were determined on the ketonic fractions of urines processed by method B. Comparing the results

[1] The subjects were seen in the Instituto de Patología Médica del Hospital Provincial of Madrid, under the direction of the Director of the Institute and Professor of Endocrinology, the late Dr. G. Marañón.

TABLE 1. EXCRETION OF DHA AND PETTENKOFER CHROMOGENS (MG./24 HRS. ±S.E.) IN NORMAL MALES AND FEMALES AND PATIENTS WITH VARIOUS ENDOCRINE DISORDERS

Diagnosis	Sex	No. of subjects	No. of determinations	DHA*	P.C.†
Normal subjects	F	10	10	0.55 (±0.08)	2.09 (±0.20)
Normal subjects	M	7	7	2.55 (±0.77)	3.80 (±0.42)
Addison's disease‡	M	4	14	0.14 (±0.02)	0.56 (±0.06)
? Adrenogenital synd.	F	1	1	3.39	4.29
Adrenogenital synd. with virilism	F	1	1	3.25	3.92
Congenital adrenogenital synd. (pseudohermaphrodism)§	F	1	1	1.21	6.87
Precocious puberty	M	1	1	0.59	1.93
Simple hirsutism	F	1	1	1.50	3.12
Simple hirsutism	F	1	1	0.58	3.16

* Dehydroepiandrosterone by method E.
† Pettenkofer chromogens determined on ketonic fractions of urine after hydrolysis by method B.
‡ Four males, before and after ACTH test (8-hr., iv.).
§ A 6-year-old girl, 45 days after unilateral adrenalectomy.

obtained by the two procedures, it can be seen that the Pettenkofer chromogens cannot always be considered to represent DHA excretion, although sometimes the differences between the results were rather small (*e.g.*, values for the normal healthy males, patients with virilism, and some cases of adrenogenital syndrome). In the normal females the output of Pettenkofer chromogens was about four times that of DHA; in the case of adrenogenital syndrome with pseudohermaphrodism it was six times greater.

In Table 2 are listed the values for Zimmermann and Pettenkofer chro-

TABLE 2. ZIMMERMANN AND PETTENKOFER CHROMOGENS IN THE KETONIC FRACTION OF URINE AFTER HYDROLYSIS AND EXTRACTION BY 4 DIFFERENT METHODS, COMPARED WITH DHA DETERMINED BY CHROMATOGRAPHIC PROCEDURE

(Average values ±S.E., in normal males and females)

Method	Color reaction	Urinary chromogens		P.C. as % of total 17-KS (Zimm.)	
		In females* (mg./liter as DHA)	In males† (mg./24 hrs. as DHA)	In females	In males
A	Zimm.	7.25 (±0.20)		45	
	Pett.	3.24 (±0.20)			
B	Zimm.	6.63 (±0.30)	13.34 (±1.63)	30	28
	Pett.	1.96 (±0.34)	3.76 (±0.53)		
C	Zimm.	1.59 (±0.13)	4.68 (±1.42)	58	62
	Pett.	0.91 (±0.07)	2.89 (±1.07)		
D	Zimm.	0.91 (±0.13)	2.89 (±0.77)	99	102
	Pett.	0.90 (±0.18)	2.95 (±0.88)		
E	Pett.	1.14 (±0.19)	2.84 (±0.86)		

* Determinations on 6 different pooled urines of females.
† Determinations on the urine of 7 males.

mogens in the urines of 7 normal males and 6 different pooled urines of normal females. The Fotherby procedure (method E) was applied to the same urines to provide a standard for the correct value of DHA. From aliquots of the normal urines the steroids were hydrolyzed and extracted by methods A, B, C and D, and the Zimmermann and Pettenkofer chromogens determined. Only method D yielded extracts in which the Zimmermann and Pettenkofer chromogens were equal and corresponded to the true DHA value. Method C, the solvolysis procedure, yielded extracts in which the levels of Pettenkofer chromogens agreed with the true values, but which contained other Zimmermann chromogens. After hydrolysis and extraction by methods A and B, not only did the Zimmermann chromogens exceed the

TABLE 3. ZIMMERMANN AND PETTENKOFER CHROMOGENS IN URINE AFTER HYDROLYSIS AND EXTRACTION BY METHOD D
(mg./liter as DHA)

Urine	Sex of subjects	Vol. of urine used (ml.)	Zimmermann chromogens*			Pettenkofer chromogens*		
			TNL	K	NK†	TNL	K	NK
Pooled	M	200	2.73	2.49	0.11	4.18	2.63	1.03
Pooled		400	2.83	2.19	0.10	4.30	2.29	0.92
Subject M.V.	M	1,000	2.42	1.46	0.07	4.12	1.81	0.70
Subject A.O.	F	1,700	0.47	0.25	0.00	0.76	0.30	0.33
Pooled	F	200	3.11	2.03	0.45		1.33	0.98
Pooled	F	200	1.00	0.37	0.03	1.83	0.18	0.24
Pooled		200	0.90	0.32	0.00	1.60	0.18	0.24
Pooled	F	200	1.73	1.18	0.13	3.34	1.27	0.78
Pooled		400	1.84	1.14	0.10	2.76	1.46	0.78

* TNL = total neutral lipid extract; K = ketonic fraction; NK = nonketonic fraction.
† Atypical yellow color.

Pettenkofer chromogens by two or four times, but the Pettenkofer chromogen values were much higher than the true values.

For determinations of DHA, even urinary extracts obtained after hydrolysis and extraction by method D had to be further purified by the Girard procedure (Table 3). The total neutral lipid extract contained two to ten times as much Pettenkofer chromogenic material as did the ketonic fraction. The excess was not accounted for in the nonketonic fraction, indicating that the chromogenic material was nonspecific. Losses of Zimmermann chromogens with Girard separation were also noted, but were smaller in degree. The data of Table 3 also show that the Pettenkofer chromogens in the nonketonic fraction may be of considerable magnitude. In extracts of urines from men, the values were about 40 per cent of those in the ketonic fraction. In extracts of urines from women, the Pettenkofer chromogens of the nonketonic fraction ranged from 50 to 133 per cent of those in the ketonic fraction. (The Pettenkofer chromogens of the nonketonic fraction produced a slightly atypical pinkish-blue color).

Methods B, C and D were used for hydrolysis of the steroids in the urine of a patient with the adrenogenital syndrome (Table 4), and an aliquot was run by the Fotherby procedure (E) to obtain the accurate DHA value. The 24-hour output of corticoids (Porter-Silber chromogens) was 1.7 mg. and that of 17-ketosteroids (17-KS) (method B) was 71 mg. By the Fotherby method, DHA excretion was 7.94 mg. per twenty-four hours. Hydrolysis and extraction of the steroids in this urine, in which the steroid excretion was not normal, by procedures C and D (Table 4) yielded extracts in which the Pettenkofer chromogen values for the ketonic fraction agreed well with the DHA values, whereas method B yielded high values. In the urine of this

TABLE 4. ZIMMERMANN AND PETTENKOFER CHROMOGENS IN THE URINE OF A BOY WITH THE ADRENOGENITAL SYNDROME*

(mg./24 hrs. as DHA)

Method	Zimmermann chromgens		Pettenkofer chromogens	
	Ketonic	Nonketonic†	Ketonic	Nonketonic
B	71.40	5.27	17.40	27.20
C	11.84	0.37	9.30	4.99
D	9.98	0.00	10.76	5.19

* See text for DHA and corticoid excretion in this patient.
† Atypical yellow color.

patient, increased amounts of Pettenkofer chromogens appeared in the nonketonic fractions of extracts prepared by methods C and D.

DISCUSSION

When dehydroepiandrosterone sulfate is hydrolyzed at elevated temperatures and low pH, as in methods A and B, 2 main artifacts are produced, namely, $\Delta^{3,5}$-androstadien-17-one and Δ^{5}-3β-chloroandrosterone. Neither of these 2 steroids is digitonin-precipitable and so they are not estimated with the DHA which is precipitated with digitonin. According to Munson *et al.* (6), dehydroepiandrosterone and its 2 artifacts from hot-acid hydrolysis can be measured by the Pettenkofer reaction. Nevertheless the results obtained after hot-acid hydrolysis by the method of Munson should not be considered as representing urinary DHA excretion. This fact, already commented on (12), is brought out very clearly when the DHA values determined by the procedure of Munson *et al.* are compared with those determined by the chromatographic method of Fotherby (Table 1). Sometimes the results obtained by both methods are not too unlike—for instance, in the group of healthy males, the average 24-hour excretion of DHA was 2.55 mg. $\pm$0.77, and Pettenkofer chromogens 3.80 mg. $\pm$0.42. Similar variations were observed among the patients (Table 1).

Burstein and Lieberman studied the solvolysis procedure for the hydrol-

ysis of ketosteroid sulfates. With the two-phase solvolysis method (16), dehydroepiandrosterone sulfate and androsterone sulfate are the 2 main urinary 17-ketosteroids hydrolyzed. The Pettenkofer-reacting compounds present in the ketonic fractions, hydrolyzed and extracted by the method (C) of Burstein and Lieberman, constituted 60 per cent of those producing the Zimmermann reaction. By methods B and A the percentages of Pettenkofer chromogens present after hot-acid hydrolysis, in the same urines, constituted 29 per cent and 45 per cent respectively of the total 17-KS. The smaller percentage of Pettenkofer chromogens yielded by these two methods corroborate the fact that only DHA and androsterone sulfate are hydrolyzed by the solvolysis procedure.

When a solution of dehydroepiandrosterone sulfate is heated at elevated temperatures under approximately neutral conditions, as in method D, another major artifact is produced, *viz*, 3,5-cyclo-androstan-6β-ol-17-one (14, 18, 19). This compound is chromogenic in the Pettenkofer reaction. Results with method D do not differ significantly (P = .99) from those obtained with the chromatographic method of Fotherby or by performing the Pettenkofer reaction on the ketonic fraction obtained after hydrolysis by the method of Burstein and Lieberman.

Since the Zimmermann chromogen values equaled the Pettenkofer chromogen values in the ketonic fractions of urinary extracts obtained after hydrolysis by method D, it would appear that dehydroepiandrosterone sulfate is the only urinary ketosteroid hydrolyzed by this procedure.

In the small series of subjects studied, the excretion of DHA in women appeared to be lower than that in men. A sex difference was also noted in the distribution of total Pettenkofer chromogens between the ketonic and nonketonic fractions; proportionally more was present in the nonketonic fractions of urines from women than of urines from men. It should be emphasized that determinations of Pettenkofer chromogens on crude neutral lipid extracts are extremely inaccurate indexes of DHA excretion. Even after the mild hydrolytic precedure, D, Pettenkofer chromogenic material was lost (in variable degree) during the Girard separation. Most of this nonspecific chromogen was nonketonic, since there was no such loss of Zimmermann chromogen.

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Letters to the Editor

THE *IN VIVO* CONVERSION OF CORTISOL-4-C^{14} TO 6β-HYDROXYCORTISOL-4-C^{14} BY THE HUMAN CIRRHOTIC LIVER*

To the Editor:

It has been suggested that 6β-hydroxycortisol,[1] originally isolated from human urine by Burstein, Dorfman and Nadel (1) is either synthesized in the human adrenal gland (1, 2), or is a metabolic product of cortisol. However, an extra-adrenal site of 6β-hydroxylation has not been directly demonstrated in the human subject. Frantz, Katz and Jailer (3) demonstrated that urinary levels of 6β-hydroxycortisol increase in the last trimester of pregnancy unrelated to any general rise of 17-hydroxycorticosteroid excretion. These authors concluded that the increased excretion reflected increased conversion of endogenous cortisol to 6β-hydroxycortisol rather than increased endogenous cortisol production *per se*. In addition, Touchstone and Blakemore have described increased urinary excretion of 6β-hydroxycortisol in 3 patients with adrenocortical hyperfunction (4). However, Ulstrom *et al.* (5) observed increased excretion of 6β-hydroxycortisol in newborn infants, a finding which may represent an hepatic mechanism for cortisol metabolism despite low levels of glucuronosyl transferase in the neonate. Axelrod and Miller described an isolated *rat* liver perfusion method which results in direct 6β-hydroxylation of desoxycorticosterone, testosterone, Δ^4-androstenedione (6) and cortisone (7). Our study was originally designed to investigate the decreased glucuronide conjugation of cortisol metabolites by the human cirrhotic liver *in vivo* (8, 9). After the rapid infusion of carrier and cortisol-4-C^{14}, unconjugated C^{14}-labeled 6β-hydroxycortisol was isolated from the alcohol:butanol-extractable steroid

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[1] The trivial names are as follows: 6β-hydroxycortisol-6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 6β-hydroxycortisol diacetate monohydrate-6β,21-diacetoxy-11β,17α-dihydroxy-4-pregnene-3,20-dione monohydrate; cortisol-11β,17α,21-trihydroxy-4-pregnene-3,20-dione; desoxycorticosterone-21-hydroxy-4-pregnene-3,20-dione; testosterone-17β-hydroxy-4-androstene-3-one; Δ^4-androstenedione-4-androstene-3,17-dione; cortisone-17α,21-dihydroxy-4-pregnene-3,11,20-trione.

fraction of hepatic vein plasma. The crystalline diacetate derivative maintained constant specific activity after recrystallization, indicating radiochemical purity, and was identified by the characteristic melting point, and infrared and sulfuric-acid spectra.

Peripheral and hepatic venous blood was obtained by catheterization. The heparinized blood samples were drawn simultaneously from both catheters at a rate of about 450 ml. per thirty minutes (200–220 ml. of plasma) immediately after the rapid infusion (eight to ten minutes) of 3.1 μC of cortisol-4-C^{14} (purified by paper chromatography; specific activity, 8.7 μC per mg.) along with 100 mg. of carrier cortisol.[2] The position of the hepatic venous catheter was verified by fluoroscopy in 3 men (J. N., A. A. and P. P.). These patients had advanced compensated Laennec's cirrhosis but demonstrated no clinical evidence of ascites, endocrine disease or renal abnormality.

The steroids were extracted from the plasma with redistilled solvents: first with chloroform for unconjugated steroids, and then successively with alcohol (to precipitate protein), *n*-butanol and *n*-butanol saturated with water, for conjugated steroids. The alcohol-butanol mixture was dried in a vacuum, dissolved in 70 per cent redistilled methanol and partitioned against hexane. The washed methanol fraction was reduced in volume (by drying in vacuum), and paper electrophoresis (using the extract from 50 ml. of plasma per paper) was performed as described previously (9). After completion of the paper electrophoresis, a nonmobile, radioactive, ultraviolet-absorbing area was observed at the starting line. Ethyl acetate eluates of this area were chromatographed successively on paper in the chloroform:formamide, toluene:propylene glycol, and finally in the benzene:50 per cent methanol (1:1) systems. The *most* polar ultraviolet-absorbing and blue tetrazolium-staining area in the last system was eluted with ethyl acetate. Radioactive measurements, performed in a liquid scintillation counter with 60 per cent efficiency for C^{14}, revealed that 0.5 (Patient J. N.), 0.6 (Patient A. A.) and 0.8 (Patient P. P) per cent of the original administered doses per 200 ml. of plasma (per patient) were associated with this polar area. Spectrophotometric analysis ((λmax. 237 EtOH) showed a total of 5.3 mg. to be present in 648 ml. of plasma (assuming a molar absorbency index of 12,300). Attempts at crystallization were unsuccessful. Acetylation was carried out at room temperature and followed by column chromatography on approximately 5.0 gm. of silica gel. The eluate, which exhibited a positive Porter-Silber reaction and maximal absorption at 237 mμ (2.1 mg.) (specific activity, 118 c.p.m. per μg.), was crystallized

[2] Cortisol was administered as the hemisuccinate diluted to 100 ml. with 0.9 per cent sodium chloride. The steroid was generously donated by The Upjohn Company, Kalamazoo, Michigan.

from ethyl acetate (specific activity, 120) and then from 95 per cent ethanol (specific activity, 126) (10). Colorless needles were formed which melted at 119–123° (uncorrected). The infrared spectrum (KBr) of the acetylated crystalline compound revealed bands identical with the spectrum of 6β-hydroxycortisol diacetate monohydrate.[3] The absorption spectrum of about 100 μg. in 3 ml. of sulfuric acid produced a violet to purple color with the following maxima at two hours: 238(O.D = 1.2); 280 (0.77); 340 (0.88); 390 (0.48); 475 (0.42). Only microgram amounts of this ultraviolet-absorbing, radioactive, polar compound were isolated from the peripheral plasma, prohibiting adequate identification.

These experiments show that 6β-hydroxycortisol appears in hepatic blood after intravenous administration of large doses of cortisol, but the data do not necessarily exclude an adrenocortical origin for *some* of the material.

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[3] The authors are indebted to the late Dr. Werner Bergmann, Department of Chemistry, Yale University, for his assistance in the interpretation of the infrared spectrum.

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ENZYMIC METHYLATION OF 2-HYDROXYESTRADIOL-17β TO 2-METHOXYESTRADIOL-17β BY HUMAN LIVER

TO THE EDITOR:

Recently, Axelrod and Goldzieher (1) reported on the methylation of 2-hydroxyestrogens by various human tissues *in vitro*. These authors made the interesting observation that human liver is unable to carry out the transmethylation reaction on 2-hydroxyestradiol-17β. This finding is in contrast to our previous experiments (2) in which it was shown conclusively that human liver does methylate 2-hydroxyestradiol-17β to 2-methoxyestradiol-17β.

It appears likely that this discrepancy may be due to the different experimental conditions used. Axelrod and Goldzieher (1) incubated finely minced liver tissue and 2-hydroxyestradiol-17β with DPN, TPN, DPNH, ATP and methionine as cofactors, whereas we (2) incubated liver slices and 2-hydroxyestradiol-17β with S-adenosylmethionine as cofactor; the latter compound was found to be essential for the enzymic methylation of 2-hydroxyestrogens. As shown by Cantoni (3), S-adenosylmethionine can be formed in the liver from adenosine triphosphate and methionine. Since homogenization of liver tissue is known to disrupt mitochondria and to liberate the enzyme adenosinetriphosphatase, the concentration of added ATP will decrease rapidly in homogenates. Therefore, in finely minced liver insufficient amounts of ATP may be available to react with methionine to produce S-adenosylmethionine. In order to check whether the negative results of Axelrod and Goldzieher (1) were in fact due to the inadequate concentration of S-adenosylmethionine, the methylation of 2-hydroxyestradiol-17β by human liver was re-investigated under various experimental conditions.

Human liver specimens (1.5–2.5 Gm.) were received at laparotomy from male patients and immediately transferred to the laboratory. The time interval between surgical removal of the specimen and the beginning of the incubation did not exceed ten minutes. Tissue slices were obtained with a razor blade by the method of Deutsch (4). Tissue brei was prepared by mincing a portion of the same liver from which slices had been cut in a

mechanical mincer previously cooled to 0° C. 2-Hydroxyestradiol-17β was synthesized by the method of Knuppen, Breuer and Pangels (5). In all experiments, 200 mg. of liver slices or 400 mg. of liver brei were incubated with 150 μg. of 2-hydroxyestradiol-17β and the appropriate cofactors in 5 ml. of Krebs phosphate saline (pH 7.4) containing 20 mM of glucose, for thirty minutes at 37° C. in air. Tissue and saline were extracted with ether-chloroform (3:1, v/v) and the extracts subjected to paper chromatography in the system formamide/monochlorobenzene. The 2-methoxyestradiol-17β formed during incubation was isolated by micro sublimation under atmospheric pressure (6) and identified by melting point (184–186° C.),

TABLE 1. ENZYMIC METHYLATION OF 2-HYDROXYESTRADIOL-17β TO 2-METHOXYESTRADIOL-17β BY HUMAN LIVER

(For details see text. Mean values are shown. The figures in brackets denote the number of experiments.)

Experimental conditions	Additions to media	2-Methoxyestradiol-17β formed (μg.)
Liver slices	None	0 [2]
	1 μM ATP+1 μM methionine	2 [4]
	1 μM S-adenosylmethionine	8 [6]
Liver mince	1 μM ATP+1 μM methionine	0 [2]
	2 μM ATP+2 μM methionine	0 [2]
	1 μM S-adenosylmethionine	8 [2]
	2 μM S-adenosylmethionine	12 [2]

Kober reaction (7, 8) (λmax. 545 mμ) and the sulfuric acid-water reaction (9) (λmax. 374 mμ, 476 mμ). The infrared spectrum (2–16 μ) obtained in KBr with a Beckman Model IR 5 infrared spectrophotometer was identical with that of an authentic sample of 2-methoxyestradiol-17β (10). Quantitative estimations were carried out with Folin-Ciocalteu's reagent (11) as described by Knuppen, Breuer and Pangels (5).

The results of the experiments are summarized in Table 1. In the presence of S-adenosylmethionine, liver slices as well as liver mince were capable of methylating 2-hydroxyestradiol-17β. When ATP and methionine were used as cofactors, enzymic methylation of 2-hydroxyestradiol-17β could be demonstrated only with liver slices and not with liver mince. These findings confirm the negative results obtained by Axelrod and Goldzieher (1) with liver mince; they also show that under certain experimental conditions human liver does methylate 2-hydroxyestradiol-17β to 2-methoxyestradiol-17β. It is highly probable that the enzyme catalyzing this reaction is identical with the O-methyl transferase which can transfer

the methyl group of S-adenosylmethionine to the 3-hydroxy group of epinephrine and other catechol amines (12).

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THYROTOXICOSIS DUE TO A SOLITARY HEPATIC METASTASIS OF THYROID CARCINOMA

To the Editor:

The ability of thyroid carcinomas to induce hyperthyroidism has been the subject of many reports. However, well documented cases are exceedingly rare (1–4).

The present case is reported both because of the extreme rarity of this clinical entity and because of the unusual fact that the entire malignant growth consisted of a single huge metastasis in which I^{131}-metabolism could therefore readily be measured.

The patient (a male) had been thyroidectomized at the age of 13 because of stenosing goiter. Another subtotal thyroidectomy for nodular goiter was performed at the age of 48. The microscopic tissue diagnosis was "proliferating struma Langhans and calcified nodular goiter." Some scattered small vesicles were found in the tumorous tissue. Both sides of the neck were afterwards irradiated. The postoperative basal metabolic rate was +21 per cent.

Ten years later (1958) an outpatient follow-up examination revealed a large mass in the upper epigastrium which the patient had noticed for over a year. Tachycardial auricular fibrillation and a small residual goiter were noted.

Laboratory findings (1958): 1) After a tracer dose of 200 μC of I^{131}, the thyroidal uptake was 8 per cent of the total dose after two hours, 7 per cent after eight hours, 7 per cent after twenty-four hours, and 7 per cent after forty-eight hours. These values corresponded roughly to the background. The conversion ratio after twenty-four hours was 86 per cent (n = 15–50 per cent). The urinary excretion rate of I^{131} after forty-eight hours was 20 per cent (n = 14–52 per cent).

2) Scanning of the neck showed very slight accumulation of I^{131} in an area of $2\frac{1}{2}$ cm. wide directly beneath the jugulum.

3) A scintigram of the hepatic region twenty-four hours after oral administration of 200 μC of I^{131} showed well marked accumulation of the tracer dose in a large area in the center of the liver.

4) A scintigram of the liver five hours after administration of 500 μC of Au^{198} showed an accumulation picture which was roughly the reverse of that obtained with I^{131}.

In December 1959 the patient was hospitalized because of violent abdominal colic. All the signs and symptoms of severe thyrotoxicosis were present—cachexia, elevated temperature, profuse sweating, tremor, diar-

rhea, tachypnea, tachycardial auricular fibrillation, and psychic excitation.

Laboratory findings (1959): The I^{131}-uptake curves for the liver and the thyroid gland are shown in Figure 1. (The liver curve offers only rough quantitative information, because an ordinary thyroid gland model was used to measure the 100 per cent uptake dose.) The conversion ratio after twenty-four hours was 68 per cent. The 24-hour urinary excretion of I^{131} was 20 per cent of total dose. Compared to the scintigram obtained eight

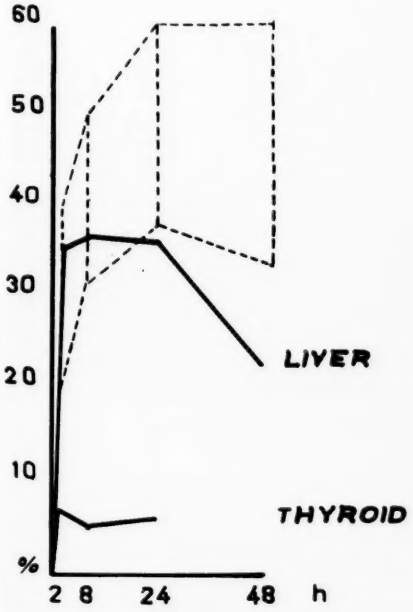

FIG. 1. I^{131} uptake (per cent of total dose) over the neck and over the liver. The standard dose was measured with the usual thyroid gland model for both curves, so quantitatively the liver values have only relative significance. (The broken lines represent the normal range of thyroidal uptake).

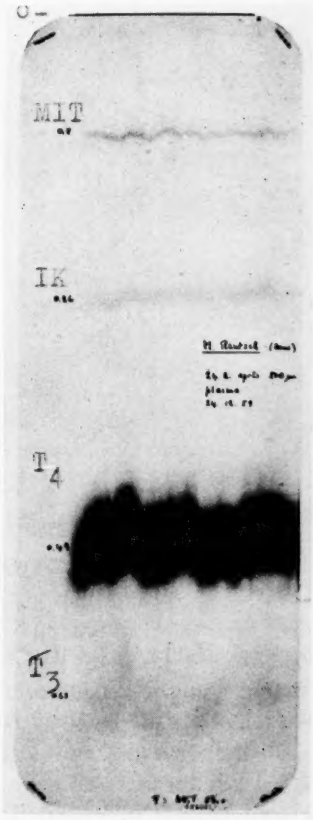

FIG. 2. Plasma chromatography (blood sample drawn twenty-four hours after oral administration of 200 μC of I^{131}). The solvent was butanol-ammonia; descending system. No abnormal compound was detectable.

months previously, scanning of the liver metastasis showed marked extension of the I^{131}-uptake area into the left hypochondrium. Paper chromatography of the plasma[1] (solvent: butanol-ammonia, descending system) from blood samples obtained twenty-four hours after administration of 200 μC of I^{131} revealed copious thyroxine formation, and traces of triiodothyronine, monoiodotyrosine and iodides (Fig. 2). The activity of serum after precipitation with trichloracetic acid was 82 per cent, with Somogyi's reagent 78 per cent, and after butanol extraction 75 per cent.

The patient died suddenly, before therapy could be started.

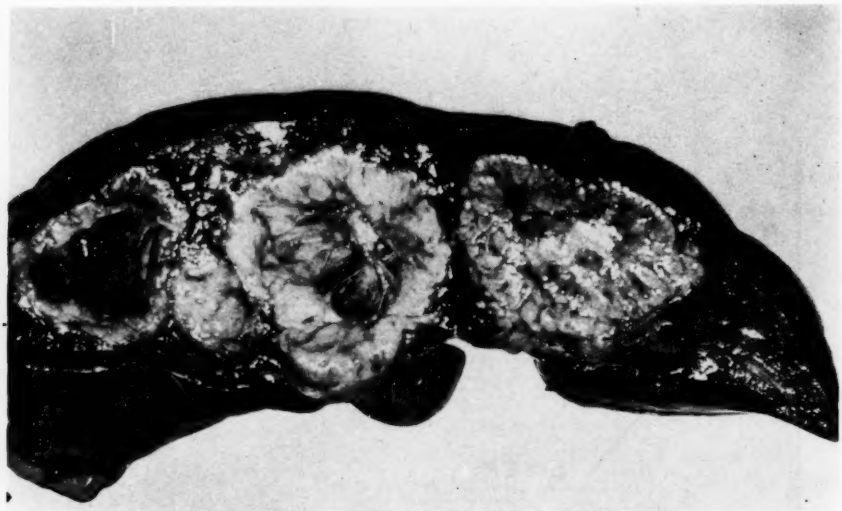

FIG. 3. Macroscopic appearance of the metastasis in the liver.

Major findings at autopsy (B. Roos, M.D., Path. Institute of Univ. of. Berne, Director Prof. B. Walthard, M.D.): The liver weighed 3300 Gm The macroscopic findings are shown Figure 3. The weight of the tumor nodules was estimated to be 2 Kg. The histologic appearance of the tumorous tissue is shown in Figure 4. The remaining liver tissue exhibited slightly advanced Laennec's cirrhosis.

Thyroid gland: On the right side there was a parenchymatous and partly cystic goitrous nodule (9 by 3 cm.), and on the left side another nodule (1 by 1.5 cm.) consisting of atrophic thyroid tissue with resting follicles (probably corresponding to the aforementioned area of minimal uptake).

[1] Carried out by Mrs. Le Marchand, Ph.D., Med. Clinic, University of Lausanne (Director: Prof. A. Vanotti, M.D.), whom we wish to thank for her valuable collaboration.

DISCUSSION

In the patient presented, the hyperthyroidism was almost entirely maintained by malignant thyroid tissue. The scintigram and the curve for I^{131} uptake indicated that the residual thyroid gland was virtually inactive Because histologically there was still a remnant of thyroid tissue with resting follicles, it must be assumed that the autonomous production of hormones in the hepatic tumor inactivated the thyroid gland by a feedback mechanism.

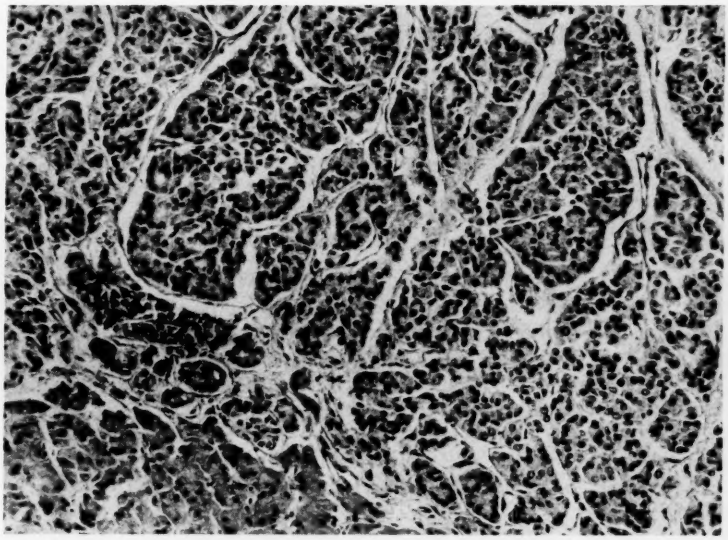

FIG. 4. Microscopic appearance of the metastasis in the liver.

Qualitatively, the hormone production of the carcinomatous cells was identical to that of normal or overactive thyroid cells. At least the chromatographic pattern of the butanol-soluble extract showed no qualitative abnormalities. However, the presence of small quantities of abnormal butanol-insoluble proteins could not be excluded with certainty.

The I^{131} uptake of the hepatic metastasis differed somewhat from that usually seen in thyroid carcinoma, inasmuch as it demonstrated a plateau-shaped curve between the second and the twenty-fourth hour. It may be assumed that the unhomogeneous structure of the tumor accounted for the particular conformation of the uptake curve.

During the twenty months after hyperthyroidism made its clinical appearance, the conversion ratio of I^{131} dropped from 36.0 to 67.7 per cent (activity more than 1300 cpm). This could be explained by the develop-

ment of some storage potential within the tumor (although colloid-containing follicles were not found) or (as would appear more reasonable) by loss of histologic, and therefore functional, differentiation. The apparently lowered hormonal activity was compensated for by enlargement of the tumorous mass, as demonstrated in successive scintigrams. Assuming a daily turnover of 10 per cent of hormonal iodine, it was calculated that at this time the tumor was about 30 times less active per unit of weight than a normal thyroid.

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The Endocrine Society

The 1962 Annual Meeting

The Forty-Fourth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday and Saturday, *June 21–22–23, 1962.*

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 22, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

The Chairman of Local Arrangements is Dr. Allan T. Kenyon.

All members are urged to make their hotel reservations immediately. The Palmer House will hold 300 bedrooms for members until May 1, 1962, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining for the A.M.A. meeting, please advise the hotel. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1962.

Everyone attending the meetings is required to register. By action of the Council, non-members will be charged a registration fee of $15.00 for any part of the scientific sessions. Members will be charged a registration fee of $5.00.

Graduate students, residents and interns, and members of the medical departments of the armed forces will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $5.00 registration fee.

Final program, membership card and advance registration forms will be sent on May 15, 1962, to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Leslie L. Bennett, Dept. of Physiology, University of California Medical Center, San Francisco 22, California, *not later than February 15, 1962.* It is imperative that the abstracts be informa-

tive and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas, graphs or extensive tables cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5″ filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Bennett.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

The 1962 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. **Evidence of scientific ability as attested by studies completed or in progress.**
2. **Recommendations from individuals familiar with the candidate and his work.**
3. **A proposed program of study.**
4. **Acceptance of the individual by the head of the department in which the Fellowship will be held.**
5. **A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.**

The Upjohn Awards

The Council of The Endocrine Society has approved a change in status of the Upjohn Scholar to that of The Upjohn Awards.

There will be two awards in the amount of $1,250 each, one for the best article in format and content printed in Endocrinology and one in The Journal of Clinical Endocrinology and Metabolism during the preceding year.

Awardees will be chosen by the Awards Committee at its regular meeting in November of each year.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

The American Thyroid Association

The next Annual Meeting of the American Thyroid Association will be held in the Hotel Roosevelt, New Orleans, Louisiana, *May 9–12, 1962.* Theodore Winship, M.D., Secretary, 430 North Michigan Avenue, Chicago 11, Illinois.

VAN METER PRIZE AWARD FOR 1962

The American Thyroid Association, Inc. again offers the Van Meter Prize Award of $500.00 to the essayist submitting the best manuscript of original and unpublished work concerning "Goiter—especially its basic cause." The studies so submitted may relate to any aspect of the thyroid gland in all of its functions in health and disease. The Award will be made at the Annual Meeting of the Association at the Roosevelt Hotel, New Orleans, Louisiana, May 9–12, 1962. A place on the program will be reserved for the winning essayist if he can attend the meeting. When more than one author's name appears on the manuscript the authors will be asked to designate a single recipient to receive the award.

The competing essays may cover either clinical or research investigations, should not exceed 3000 words in length and must be presented in English. Duplicate, typewritten copies, double spaced, should be sent to the Secretary: Theodore Winship, M.D., 430 N. Michigan Ave., Chicago 11, Illinois, *not later than January 1, 1962.* The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

Symposium on Basic Problems in Neoplastic Disease

Columbia University College of Physicians and Surgeons is sponsoring a three-day Symposium, titled "Basic Problems in Neoplastic Disease," to be held on *March 12, 13 and 14, 1962.* This Symposium will commemorate the 50th Anniversary of the Institute of Cancer Research at Columbia University and the 10th Anniversary of its affiliated clinical facility, The Francis Delafield Hospital.

An outstanding group of scientists from the United States and Abroad will participate in sessions titled as follows: Nucleic Acid Structure and Synthesis; Viral and Genetic Studies; Protein Synthesis; Antibody Structure and Function. In addition, sessions devoted to clinical aspects of the biochemistry, pathological-physiology, morphology, and therapy of cancer will be held.

The symposium is open without fee to all interested workers in this field. For details concerning the meeting, including application to attend sessions, write to the Institute for Cancer Research, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York.

Symposium on Steroidal Enol and Phenol Ethers

The First Symposium on Steroidal Enol and Phenol Ethers in the Clinic and in Biology, sponsored by the Italian Society of Endocrinology, will be held in Rome on *December 11, 1961.* Inquiries should be addressed to the Symposium Secretariat: Istituto di Patologia Speciale Medica, Policlinico Umberto I°, Rome, Italy.

Convention of Pacific Coast Fertility Society

The Tenth Annual Convention of the Pacific Coast Fertility Society will be held at the El Mirador Hotel in Palm Springs, California, *November 9–12, 1961.* Guest speakers of the highest national and international reputation will be on the program. Address inquiries to the Secretary,Gregory Smith, M.D., 909 Hyde Street, San Francisco 9, California.